# Denaturation of Proteins by SDS and Tetraalkylammonium Dodecyl Sulfates

# Denaturation of Proteins by SDS and by Tetra-alkylammonium Dodecyl Sulfates

Andrew Lee, Sindy K. Y. Tang, Charles R. Mace, and George M. Whitesides*

*Department of Chemistry and Chemical Biology, Harvard University*

*12 Oxford St., Cambridge, MA, U.S.A.*

* Authors to whom correspondence should be addressed

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**ABSTRACT**

This paper describes the use of capillary electrophoresis (CE) to examine the influence of different cations ($C^+$; $C^+ = Na^+$ and tetra-*n*-alkylammonium, $NR_4^+$, where R = Me, Et, Pr, Bu) on the rates of denaturation of bovine carbonic anhydrase II (BCA) in the presence of the anionic surfactant dodecylsulfate ($DS^-$). Analysis of the denaturation of BCA in solutions of $Na^+DS^-$ and $NR_4^+DS^-$ (in Tris-Gly buffer) indicated that the rates of formation of complexes of denatured BCA with $DS^-$ ($BCA^D$-$DS^-_{n,sat}$) are indistinguishable and independent of the cation *below the critical micellar concentration (cmc)*, and independent of the total concentration of $DS^-$ *above the cmc*. At concentrations of $C^+DS^-$ above the cmc, BCA denatured with rates that depended on the cation; the rates decreased by a factor > $10^4$, in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$ – the same order as the values of cmc (which decrease from 4.0 mM for $Na^+DS^-$ to 0.9 mM for $NBu_4^+DS^-$ in Tris-Gly buffer). The relationship between values of cmc and rates of formation of $BCA^D$-$DS^-_{n,sat}$ suggested that the kinetics of denaturation of BCA involve the association of this protein with monomeric $DS^-$, rather than with micelles of $(C^+DS^-)_n$. A less-detailed survey of seven other proteins (α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, carboxypeptidase B, creatine phosphokinase, myoglobin, and ubiquitin) showed that the difference between $Na^+DS^-$ and $NR_4^+DS^-$ observed with BCA was not general. Instead, the influence of $NR_4^+$ on the association of $DS^-$ with these proteins depended on the protein. The selection of cation contributed to the properties (including composition, electrophoretic mobility, and partitioning behavior in aqueous two-phase systems) of aggregates of denatured protein and $DS^-$. These results suggest

that variation in the behavior of $NR_4^+DS^-$ with changes in R may be exploited in methods for analyzing and separating mixtures of proteins.

## INTRODUCTION

This paper analyzes the interaction between proteins and anionic surfactants ($C^+DS^-$) that consist of dodecylsulfate ($DS^-$) paired with the cations $Na^+$, tetramethylammonium ($NMe_4^+$), tetraethylammonium ($NEt_4^+$), tetra-*n*-propylammonium ($NPr_4^+$), or tetra-*n*-butylammonium ($NBu_4^+$). Using capillary electrophoresis (CE), we analyzed the formation of complexes of $DS^-$ with bovine carbonic anhydrase II (BCA; E.C. 4.2.1.1) in detail. This reaction involves the association of *n* equivalents of $DS^-$ to BCA, and results in complexes of denatured protein and $DS^-$, $BCA^D$-$DS^-_{n,sat}$, where *n,sat* is the stoichiometry of $DS^-$ bound to BCA at saturation in solutions containing an excess of $DS^-$ (as observed when BCA is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; *n,sat* is approximately 150 for BCA)[1] (Eq. 1).

$$BCA + n\ DS^- \rightarrow BCA^D\text{-}DS^-_{n,sat} \qquad (1)$$

The rates of denaturation of BCA depended on $C^+$. At concentrations of $C^+DS^-$ below the critical micellar concentration (cmc), the rates were independent of the cation, and increased with the concentration of $DS^-$ (log *k* was approximately proportional to $[DS^-]$ below the cmc). At concentrations above the cmc however, rates of formation of $BCA^D$-$DS^-_{n,sat}$ were independent of increases in the total concentration of $C^+DS^-$ and depended on the cation, decreasing in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. This order is the same as the order of values of cmc, and suggests that the rates of formation of $BCA^D$-$DS^-_{n,sat}$ above the cmc depend on the concentration of monomeric $DS^-$. This order is also, of course, the order of increasing size and hydrophobicity

(described by increasing values of log *P* for the water-octanol partitioning of $NR_4^+$: -2.7, -2.1, -1.3, 0.4).[2, 3]

We also surveyed – albeit in less detail – seven other proteins (α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, carboxypeptidase B, creatine phosphokinase, myoglobin, and ubiquitin) for their interactions with $C^+DS^-$. The influence of the cation on formation of aggregates between protein and $DS^-$ varied with the protein. For some (e.g., ovalbumin), the rate of formation of protein-$DS^-_n$ was indistinguishable in solutions of $Na^+DS^-$ and $NR_4^+DS^-$. For others (e.g., carboxypeptidase B; CPB), the cation influenced the kinetics of formation and composition of aggregates of protein and $DS^-$.

**Motivation.** Treatments of proteins with surfactants are widely used in biotechnology, analytical biochemistry, and medicine.[4-11] Examples include the ubiquitous application of SDS in SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis), the use of surfactants in formulations of biopharmaceuticals (to prevent the aggregation of proteins and adsorption of proteins to surfaces), and the use of surfactants in cleaning, disinfecting, and sterilizing.[12-16] Our initial interest was in SDS-PAGE. This technique remains an integral part of protein biochemistry, but obvious questions – why dodecyl sulfate, rather than some other surfactant? why sodium ions? – seem never to have been systematically addressed. We hoped that such an examination would increase the number of knobs that could be turned by biochemists in manipulating the interactions among proteins and surfactants.

In the preparation of samples for SDS-PAGE, the combination of temperature (100 °C), surfactant (~70 mM SDS >> 4.0 mM, the cmc in Tris-Gly buffer, one that is commonly used in SDS-PAGE), and disulfide reducing agent unfolds most globular

proteins and generates aggregates of denatured protein and $DS^-$ (which we write as protein$^D$-$DS^-_{n,sat}$).[17, 18] The stoichiometry of binding is roughly independent of the amino-acid sequence and structure of the native protein (~1.4 g of SDS per 1 g of protein, or approximately one equivalent of $DS^-$ for every two amino acid residues).[19, 20] This feature enables the resolution of proteins by PAGE according to molecular weight. Exceptions that show anomalous migration include post-translationally modified proteins, membrane-associated or membrane-bound proteins, and proteins with very high or low isoelectric points.[21-24] Many elements of the interaction of $DS^-$ with proteins remain unclear. Details about the sites of association, the relative importance of hydrophobic and electrostatic contributions to binding, and the mechanism by which $DS^-$ induces conformational change, are incompletely understood despite extensive investigation.[10, 20, 24-28] Whether or how $Na^+$ (or other cations in buffer solutions of $Na^+DS^-$ and protein) participates is not known.

Our primary objective in this study was to compare the influence of cations having a range of hydrophobicity ($C^+$ = $Na^+$; $NR_4^+$, R = Me, Et, Pr, Bu). To identify the effect of $NR_4^+$ on the formation of protein-$DS^-_n$, we analyzed BCA and several additional proteins using CE, in solutions of $C^+DS^-$. Zana et.al. compared the properties (cmc, aggregation number ($N$), degree of ionization ($\alpha^0$), microviscosity, micropolarity) of $Na^+DS^-$ and $NR_4^+DS^-$ in unbuffered aqueous solution; the results suggested that the assembly of $DS^-$ into micelles was more favorable for $NEt_4^+DS^-$, $NPr_4^+DS^-$, and $NBu_4^+DS^-$ than for $Na^+DS^-$.[3, 29-33] Trends in the values for cmc and $\alpha^0$ implicated the binding of $NR_4^+$ to micelles with affinity greater than $Na^+$.[29-32] We anticipated that the

variation of the cation from $Na^+$ to $NR_4^+$ would provide a way to manipulate interactions between $DS^-$ and proteins, and to probe the reaction of $DS^-$ with BCA.

**Interaction of Proteins with $Na^+DS^-$: Concentrations Below or Near the Cmc.** Isotherms describing the binding of $Na^+DS^-$ to proteins have identified two types of association between protein and $DS^-$: binding of $Na^+DS^-$ at low stoichiometric equivalencies at concentrations below the cmc, and cooperative binding of $Na^+DS^-$ at high equivalencies at concentrations near or above the cmc. At concentrations ≤ 1 mM, $Na^+DS^-$ binds to BSA (~6 equivalents) and cutinase (~14 equivalents) without significant change to the structure of the protein; mushroom tyrosinase, creatine phosphokinase, ubiquitin, and soybean peroxidase are other examples of proteins that show this type of binding.[34-39] Discrete stoichiometries of binding, at concentrations below the cmc, suggest the binding of individual molecules of $Na^+DS^-$ (rather than micelles) with high affinity. At concentrations of $Na^+DS^-$ near the cmc, cooperative binding of large numbers of $Na^+DS^-$ is accompanied (for most proteins) by the loss of native tertiary structure, and by changes in secondary structure of the native protein.[20, 40]

The concentration of $Na^+DS^-$ that causes denaturation varies with the protein. For myoglobin[37, 41] and ferrocytochrome C[42], binding of monomeric $Na^+DS^-$ at concentrations (≤ 1 mM) below the cmc is sufficient to cause denaturation. Proteins such as BCA and creatine phosphokinase however, do not bind $Na^+DS^-$ and remain folded until concentrations are near or above the cmc (4.0 mM in Tris-Gly).[25, 38] Rapid denaturation, at concentrations just below the cmc, suggests a mechanism in which the protein serves as a template for the condensation of $DS^-$, and a site of nucleation for micelles of $Na^+DS^-$. This hypothesis is consistent with the adsorption of $Na^+DS^-$ onto self-assembled

monolayers of $CH_3$-terminated alkanethiols on Au, at concentrations below the cmc (by a factor of ~10).[43, 44] Factors that may contribute to the resistance of some proteins (e.g., superoxide dismutase, avidin) to denaturation at 22 °C, even at concentrations well above the cmc, include electrostatic repulsion of highly negatively charged proteins (e.g., pepsin) or structural features of the native proteins (e.g., extensive β-sheet structure).[22, 45]

We have studied the association of $Na^+DS^-$ to ubiquitin (UBI), an 8.6-kDa single-chain protein, in detail.[46] Upon equilibration in solutions of $Na^+DS^-$ (0.05 - 10.00 mM in Tris-Gly buffer, 22 °C), UBI forms six groups of complexes (UBI-$SDS_n$) distinguishable by CE. Stoichiometries were approximately defined for several complexes (UBI-$SDS_{\sim 11}$, UBI-$SDS_{\sim 25}$, UBI-$SDS_{\sim 33}$).[47] Identification of these complexes suggested that the denaturation of UBI (ultimately leading to UBI-$SDS_{\sim 42}$) proceeds through several stable intermediates. UBI-$SDS_{\sim 11}$ retained the secondary structure of native UBI, and the stoichiometry of binding was approximately equivalent to the number of cationic residues (UBI has seven lysine and four arginine residues). This observation was consistent with the hypothesis that $Na^+DS^-$ binds to proteins initially at the sites of cationic residues (inferred from similar observations in other proteins, e.g., lysozyme).[10, 34, 48, 495] Investigation of UBI-$SDS_{\sim 11}$ by NMR however found that SDS associates primarily at residues with hydrophobic side chains located near positively charged amino acid residues, but not at the lysine and arginine residues themselves.[50]

**Interactions of Proteins with Micelles of $Na^+DS^-$.** Solutions of $Na^+DS^-$ above the cmc contain both monomeric $Na^+DS^-$ and micelles of $Na^+DS^-$. Above the cmc, an increase in the concentration of $Na^+DS^-$ leads to greater numbers of micelles (as well as changes in the size and shape of the micelles – discussed below), but the concentration of

monomeric $Na^+DS^-$ remains constant.[51-57] Interactions between native proteins and micelles of $Na^+DS^-$ have been inferred from the analysis of proteins at concentrations of $Na^+DS^-$ above the cmc. Rates of unfolding for S6 (a 101-residue polypeptide)[28] increased (from $10^2$ $s^{-1}$ to $10^4$ $s^{-1}$) with concentrations of $Na^+DS^-$ in the range 10 - 100 mM – a range well above the cmc – and supported the conclusion that S6 unfolds upon direct association with a micelle of $Na^+DS^-$.[28,37, 41] At concentrations of $Na^+DS^- \geq 200$ mM (at which rod-like micelles begin to predominate), rates of unfolding increased with a power-law dependence on the concentration of $Na^+DS^-$ ($k_{obs} \alpha [Na^+DS^-]^{3.5}$), suggesting that rod-like micelles are more reactive than spherical micelles are in denaturing S6.[58, 59]

**Influence of Cations on the Formation of Micelles of $DS^-$.** The assembly of anionic surfactants into micelles is opposed by the electrostatic repulsion of negatively charged headgroups.[51, 60] Participation of cations, by association with the surface of the micelles and screening of negatively charged headgroups, is essential to the formation of micelles of $DS^-$.[61] The association of $Na^+$ to micelles of $DS^-$ has been inferred from the conductivity of solutions of $Na^+DS^-$.[23,62] Data from this method suggest a value of 0.23 for the degree of ionization ($\alpha^0$) for micelles of $Na^+DS^-$, and therefore suggest a value of 0.77 for the degree of association ($1 - \alpha^0$) of $Na^+$ to micellar $DS^-$ (the degree of association is the fraction of electrostatic charge of the surfactant headgroup neutralized by counterions bound at the surface of the micelle).[29] Other techniques corroborate the binding of $Na^+$ to micelles of $DS^-$.[63-68] Increasing concentrations of $Na^+$ (e.g., by the addition of NaCl) result in lower values of cmc; the cmc is inversely proportional to ($cmc_0 + [Na^+]_{added}$), where $cmc_0$ is the cmc in the absence of additional $Na^+$.[69]

Association of cations also influences the size and shape of micelles. With increasing [$Na^+$] (from 20 to 140 mM), values of aggregation number (*N* , the average number of surfactant molecules comprising an individual micelle) for micelles of $Na^+DS^-$ increase from 60 to 90, and suggest the tighter packing of $DS^-$, enabled by the screening of $SO_4^-$ headgroups by $Na^+$.[54, 55, 70] Rod-like micelles require tighter packing of headgroups and greater screening by $Na^+$ than in spherical micelles; the transition from spherical to rod-like micelles is observed at 140 - 200 mM $Na^+DS^-$.[56, 57, 61, 71-73]

**Properties of $NR_4^+DS^-$.** Zana investigated the properties of $Na^+DS^-$ and $NR_4^+DS^-$ prepared by ion-exchange chromatography.[29] Values of cmc in unbuffered aqueous solution decreased from 8.0 to 1.2 mM, in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. This order was also found in the values for the degree of ionization ($\alpha^0$) for micelles of $Na^+DS^-$ and $NR_4^+DS^-$, decreasing from 0.23 for $Na^+DS^-$ to 0.17 for $NBu_4^+DS^-$. These trends suggested that binding of $NR_4^+$ to micelles of $DS^-$ is more favorable than binding of $Na^+$ (presumably due to hydrophobic interactions). Values of the aggregation *N* for $Na^+DS^-$ and for each $NR_4^+DS^-$ were in the range of 60 - 100, indicating micelles that were spherical and approximately the same size, regardless of the cation.[29] These data indicated that micelles of $NPr_4^+DS^-$ and $NBu_4^+DS^-$ differ from micelles of $Na^+DS^-$ in three ways: (i) the surface density of net negative charge, due to differences in the density of cations bound to the surface of micelles; (ii) hydrophobic interactions between $NR_4^+$ and $DS^-$ at the surface of micelles; (iii) hydrophobic interactions among $NR_4^+$.[29-31,74]

**EXPERIMENTAL DESIGN.**

**Selection of $C^+DS^-$.** We selected $Na^+DS^-$ and the series of surfactants $NR_4^+DS^-$ for four reasons: (i) The properties of $Na^+DS^-$ and $NR_4^+DS^-$ (i.e., cmc, $\alpha^0$) systematically depend on the hydrophobicity of $NR_4^+$. (ii) The preparation of $NR_4^+DS^-$ from $Na^+DS^-$ by ion-exchange chromatography is straightforward. (iii) The optical properties of these systems make analysis straightforward in CE with a UV-absorbance detector: $Na^+DS^-$ and $NR_4^+DS^-$ are transparent at 214 nm and do not interfere with the detection of proteins, in either native form or in complexes with $DS^-$. (iv) The complete series of surfactants – $Na^+DS^-$ and $NR_4^+DS^-$ – is soluble in Tris-Gly buffer at 22 °C over a range of concentrations (0 - 100 mM) that is convenient for analyzing the association of $DS^-$ with proteins.

We chose to study $Na^+DS^-$ and $NR_4^+DS^-$, rather than the series of surfactants consisting of $DS^-$ and alkali metal ions, because differences in the properties of $C^+DS^-$ ($C^+ = Li^+$, $Na^+$, $K^+$, or $Cs^+$) are relatively small as $C^+$ is varied. For example, as $C^+$ is changed from $Li^+$ to $Cs^+$, values of cmc decrease from 8.9 mM to 6.1 mM (in unbuffered solution at 25 °C);[75] the range of values for cmc is broader for $NR_4^+DS^-$ and $Na^+DS^-$ (1.2 - 8.0 mM)[29, 76].

**Solutions of $C^+DS^-$ Tris-Gly Buffer.** Values of the cmc for $NR_4^+DS^-$ in buffered solutions have not been reported. We determined these values for $Na^+DS^-$ and $NR_4^+DS^-$ in Tris-Gly buffer with established methods using fluorescent probes (pyrene and 8-anilinonapthalenesulfonate),[77-79] or UV-absorbing probes (e.g., 2-naphthalenecarbinol) in CE experiments.[46, 80] CE was also useful for characterizing the electrophoretic mobility of micelles of $Na^+DS^-$ and $NR_4^+DS^-$. The method used to analyze the

partitioning of UV-absorbing probes into micelles is described in the Supporting Information.

Concentrations of $C^+DS^-$ in the range 0.0 - 10.0 mM allowed us to analyze proteins in solutions below and above the cmc. Previous work showed that this range is convenient for analyzing reactions of $Na^+DS^-$ with proteins at 22 °C; for many proteins, the formation of protein$^D$-$DS^-_{n,sat}$ is rapid at concentrations of $Na^+DS^-$ within this range.[25, 38] Concentrations of $Na^+DS^-$ and $NR_4^+DS^-$ were also low enough (<< 200 mM) to justify the assumption that micelles of $Na^+DS^-$ and $NR_4^+DS^-$ were spherical and approximately the same size (values of $N$ in the range 60-100).[29, 54, 81]

We analyzed proteins in solutions of $C^+DS^-$ in Tris-Gly buffer (25 mM Tris-192 mM glycine, pH = 8.4), a buffer that is typically used in SDS-PAGE. The ions in Tris-Gly buffer are the cation of tris $(HOCH_2CH_2)_3NH^+$, 8.3 mM), and a mixture of the anion of glycine ($H_2NCH_2CO_2^-$, 8.3 mM) and the zwitterion of glycine ($H_3N^+CH_2CO_2^-$, 184 mM) (concentrations were determined by calculation, using values of p$K_a$ reported for Tris - 8.06, and for glycine - 2.3, 4.3, 7.7, 9.7).[82] It is possible that buffer cations participate in the interactions between protein and $DS^-$. The buffer cations however are not responsible for data distinguishing the influence of $C^+$ ($Na^+$ and $NR_4^+$) on the formation of protein-$DS^-_n$, since all of our experiments used Tris-Gly at the same concentration.[83] We did not test buffers other than Tris-Gly (e.g., phosphate, carbonate, or borate).

**BCA as a Model Protein.** We and others have used BCA extensively as a model protein in biophysical studies aimed at protein folding, protein-surfactant interactions, and rational ligand design.[27, 84-86] BCA is monomeric and has no disulfide bonds.[84, 85]

Native BCA has both α-helical and β-sheet elements; a nine-stranded β-sheet forms the core of the protein. The electrophoretic migration of BCA in SDS-PAGE is consistent with its molecular weight (29.1-kDa). Based on stoichiometries of binding that are typical for proteins analyzed in SDS-PAGE, we assume that the formation of $BCA^{D}$-$DS^{-}_{n,sat}$ involves the association of ~150 equivalents of $DS^{-}$ (i.e., $n$ ~ 150, estimated from the binding of ~1.4 g of $Na^{+}DS^{-}$ for every 1 g of protein).[19]

**Analysis of the Formation of $BCA^{D}$-$DS^{-}_{n,sat}$ by CE.** We used CE for its ability to detect and characterize the association of $DS^{-}$ with proteins. UV absorbance (214 nm) of the amides of the polypeptide backbone allowed us to detect BCA in all possible forms (i.e., native or denatured in complexes with $DS^{-}$), without complication from $DS^{-}$ or $C^{+}$, which are transparent in the UV.

CE resolves analytes according to their electrophoretic mobility – the ratio of electrostatic charge to hydrodynamic drag – in free solution. Equation 2 is an expression for the mobility of native protein ($\mu_N$) having net electrostatic charge $Z_0$ and mass $M_0$; $\alpha$ is a constant that depends on the shape of the analyte, often estimated as 2/3 for proteins, and $C_p$ is a constant.[87]

$$\mu_N = C_p \frac{Z_0}{(M_0)^{\alpha}} \qquad (2)$$

The binding of $DS^{-}$ (and formation of protein-$DS^{-}_{n}$) results in values of electrophoretic mobility higher than $\mu_N$. Equation 3 shows the dependence of the mobility of protein-$DS^{-}_{n}$ ($\mu_{den}$) on the electrostatic charge and mass of $DS^{-}$ ($\Delta Z_{DS^-}$ and

$M_{DS-}$) and a correction factor ($C_{\psi,n}$) for the non-linear dependence of $\mu$ on $Z$ at high surface potentials ( > 25 mV).[88, 89]

$$\mu_{den} = C_p C_\psi \frac{Z_0 + n\Delta Z_{DS^-}}{(M_0 + n \cdot M_{DS^-})^\alpha} \qquad (3)$$

Values of $\mu_{den}$ are greater than $\mu_N$ because the increase in negative charge is significant (upon binding of $DS^-$ at a ratio of 1 $DS^-$ for every 2 amino acid residues) and is only partially compensated by an increase in hydrodynamic drag (due to an increase in mass by a factor of ~2.4).[90] The amount of negative charged contributed by the binding of each equivalent of $DS^-$ ( $\Delta Z_{DS-}$ ) is however less than one unit, and probably ~0.9, due to charge regulation.[91] Furthermore, Eq. 3 does not include contributions from cations associated with protein-$DS^-_n$; the association of $Na^+$ has typically been ignored in the analysis of $\mu_{den}$ (expressions that include contributions from $C^+$ are discussed in the Supporting Information). In practice, values of $\mu_{den}$ for protein-$DS^-_n$ are typically 18 - 22 $cm^2\ kV^{-1}\ min^{-1}$.

**Generation and Analysis of Protein-$DS^-_n$ by SurfactantCE (SurfCE).**

"SurfCE" is a technique we have developed for surveying the association of ionic surfactants to proteins. SurfCE analyzes reactions of proteins with $C^+DS^-$ carried out within capillaries. Samples of protein, after injection (in their native form) into capillaries filled with solutions of running buffer and $C^+DS^-$, migrate through solutions of $C^+DS^-$ during electrophoresis. Conversion of native protein to protein-$DS^-_n$ results in peaks reflecting both the composition of protein-$DS^-_n$ and the time (and therefore rate) of

its formation. SurfCE identifies concentrations of $C^+DS^-$ that cause formation of protein-$DS^-_n$ within the retention time of proteins in the capillary (~3 min. in most of our experiments, which used an applied voltage of 30 kV with 60-cm capillaries having a distance of 50 cm from the inlet to detector). Data from SurfCE can develop qualitative, and in some cases quantitative, information about the kinetics of reaction.

Electropherograms showing peaks only for native protein are the result of either (i) an equilibrium that favors the native form (a thermodynamic outcome), or (ii) slow reactions that require more time than the duration of the experiment (a kinetic outcome). Other methods were necessary to analyze reactions that were too slow for analysis by SurfCE (e.g., Capillary Zone Electrophoresis, or measurement of folded BCA with the fluorescence of dansyl amide).

**Other Proteins.** We surveyed the interactions of $C^+DS^-$ with proteins other than BCA by SurfCE. These experiments allowed us to examine the possibility that the interactions of BCA with $NR_4^+DS^-$ were exceptional, as well as identify general trends in the influence of $NR_4^+$ on the formation of complexes of protein and $DS^-$. We selected seven proteins that met three criteria: (i) values of molecular weight and p*I* that allow for convenient analysis by CE; (ii) absence of disulfide bonds that could complicate the formation of protein$^D$-$DS^-_{n,sat}$; (iii) commercial availability. The properties of these proteins are summarized in Table 2 of the Results.

## RESULTS AND DISCUSSION.

**Preparation of $NR_4^+DS^-$, from $Na^+DS^-$, by cation-exchange.** We prepared the surfactants $Na^+DS^-$, $NMe_4^+DS^-$, $NEt_4^+DS^-$, $NPr_4^+DS^-$, and $NBu_4^+DS^-$ by using a three-step

procedure: (i) re-crystallization of $Na^+DS^-$ from ethanol (twice); (ii) replacement of $Na^+$ with $H_3O^+$ by cation-exchange chromatography; (iii) reaction of $H_3O^+DS^-$ with standard solutions of $C^+OH^-$ (NaOH, $NMe_4OH$, $NEt_4OH$, $NPr_4OH$, or $NBu_4OH$).[92]

We used this procedure to prepare samples of $Na^+DS^-$ and $NR_4^+DS^-$ derived from a single batch of re-crystallized $Na^+DS^-$. Samples of $Na^+DS^-$ prepared by two different methods – re-crystallization (step (i) only) or by ion-exchange (steps (i)-(iii)) – showed agreement in values of cmc, $^1H$ NMR spectra, and data characterizing the interaction of $Na^+DS^-$ with proteins. This agreement suggested that the procedure for replacing $Na^+$ with $C^+$ did not alter samples of $DS^-$ in unintended ways (e.g., by hydrolyzing $C_{12}H_{25}$-$OSO_3^-$ groups, or adding impurities from the resin). We prepared stock solutions of each surfactant in Tris-Gly buffer (25 mM Tris-25 mM glycine, pH = 8.4).

**Measurement of Cmc.** Methods for estimating the cmc, by detecting micelles with pyrene or ANS as fluorescent probes, have been described in detail previously.[77, 79] We analyzed the fluorescence spectrum of pyrene (2 μM) in solutions of $Na^+DS^-$ and $NR_4^+DS^-$ in Tris-Gly buffer to determine the cmc of each surfactant. Values of cmc are reported in Table 1 (examples of fluorescence spectra and analysis are in the Supporting Information: Fig. S1A-C).

We confirmed these values with a second method. We analyzed the partitioning of 2-naphthalenecarbinol (naph) into micelles of $C^+DS^-$ by CE. Values of electrophoretic mobility for naph ($\mu_{naph}$) indicated the rapid equilibration of naph between aqueous buffer and negatively charged micelles of $DS^-$. Estimates of the cmc, obtained by extrapolating curves relating $\mu_{naph}$ to $[C^+DS^-]$, were within 0.3 mM of values determined by the fluorescence of pyrene (also in Table 1).[93]

**Table 1.** Values of cmc of *n*-$C_{12}H_{25}SO_4^-$ as a Function of the Cation

| | | cmc (mM) | | |
|---|---|---|---|---|
| **Cation** | **Preparation** | **Tris-Gly[a] (fluorescence[b])** | **Tris-Gly[a] (CE[c])** | **$H_2O$[d] (conductivity)** |
| $Na^+$ | C[e] | 4.0 | 4.3 | 8.0 |
| $Na^+$ | IE[f] | 4.1 | 4.3 | 8.0 |
| $NMe_4^+$ | IE | 3.8 | 4.0 | 5.4 |
| $NEt_4^+$ | IE | 2.6 | 2.9 | 3.7 |
| $NPr_4^+$ | IE | 1.7 | 2.0 | 2.2 |
| $NBu_4^+$ | IE | 0.9 | 1.2 | 1.2 |

[a] Tris-Gly buffer: 25 mM Tris-192 mM glycine, pH = 8.4. [b] Determined by analyzing the fluorescence spectrum of pyrene in solutions of surfactant (data in the Supporting Information: Figure S1). [c] Determined by analyzing the mobility of 2-naphthalenecarbinol, in solutions of surfactant, by CE (data in Fig. S1D). [d] Values taken from Ref[29]. [e] Re-crystallized twice from ethanol. [f] Prepared by ion-exchange (as described in the text) from a single batch of recrystallized $Na^+DS^-$.

**Replacement of $Na^+$ with $NR_4^+$ decreases the cmc of $DS^-$ in Tris-Gly.** Values of cmc in Tris-Gly buffer (Table 1), follow the order $Na^+ > NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. The trend is the same as the trend in unbuffered aqueous solution (last column in Table 1, taken from Ref[29]). It suggests that the binding of cations to micelles of $DS^-$ is more favorable for increasingly hydrophobic cations, since the formation of micelles depends upon the screening of negatively charged $R\text{-}OSO_3^-$ headgroups by cations. Values of cmc are lower for each surfactant in Tris-Gly than in unbuffered solution, and are probably the result of additional screening of $DS^-$ by the buffer ions in Tris-Gly (ionic strength ~10 mM) on micelle formation.

Zana also identified trends in $\alpha^0$ and $N$ for $NR_4^+DS^-$ in unbuffered solution;[29] these trends probably hold in solutions of Tris-Gly as well. We assumed that micelles of $Na^+DS^-$ and $NR_4^+DS^-$ are spherical and approximately the same size (aggregates of 60 - 90 equivalents of $DS^-$)[29] at concentrations through 10.0 mM, and that the amount of cation associated to micelles of $DS^-$ increases in the order $Na^+ < NMe_4^+ < NEt_4^+ < NPr_4^+ < NBu_4^+$.

**Hypotheses Rationalizing the Influence of $C^+$ on Reactions of $DS^-$ with Proteins.** The trend in the cmc of $C^+DS^-$ describes the influence of $C^+$ on the formation of micelles of $DS^-$; it also suggests several hypotheses for how $C^+$ may influence the interaction of $DS^-$ with proteins. We considered three cases:

(i) If denaturation is induced by the association of monomeric $DS^-$ to proteins, rates of denaturation in solutions below the cmc should increase with the concentration of $DS^-$, but should be independent of $C^+$. Equation 4a describes a process in which *a* equivalents of monomeric $DS^-$ ($DS^-_{mon}$) associate with the protein in the initial step,

causing the protein to partially unfold, leading to cooperative association of $DS^-$ and denaturation. Rates of denaturation should not increase with the concentration of $C^+DS^-$ above the cmc, since additional $DS^-$ above the cmc is present only in micelles. Since values of cmc indicate the maximum possible concentration of monomeric $DS^-$ in a solution of $C^+DS^-$, the hypothesis of Eq. 4a, and the values of cmc in Table 1, predict *rates of denaturation in the order* $Na^+ > NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$ *in solutions above the cmc.*

$$\text{protein} + a\text{-}DS^-_{mon} \rightleftharpoons [\text{protein-}(DS^-_{mon})_a]^{\ddagger} \xrightarrow{DS^-} \text{protein}^D\text{-}(DS^-)_{n,sat} \qquad (4a)$$

(ii) If denaturation is induced by the cooperative condensation of $DS^-$ onto the protein (Eq. 4b) – in a process similar to the formation of $C^+DS^-$ micelles – rapid denaturation should occur at concentrations of $C^+DS^-$ near the cmc. According to this hypothesis, denaturation would be rapid near the cmc because $C^+$ would promote the aggregation of $DS^-$ onto the protein (the protein provides a template), in the same way that $C^+$ promotes the aggregation of $DS^-$ into micelles. In Eq. 4b, [protein-$(DS^-_{mic})_b$] is the structure of protein as it nucleates the condensation of *b*-equivalents of $DS^-_{mon}$ and the formation of a micelle of $DS^-$. This hypothesis predicts rapid denaturation at concentrations of $C^+DS^-$ that follow the trend in value of cmc and *therefore at concentrations that increase in the order* $NBu_4^+ < NPr_4^+ < NEt_4^+ < NMe_4^+ < Na^+$.

$$\text{protein} + b\text{-}DS^-_{mon} \longrightarrow [\text{protein-}(DS^-_{mic})_b]^{\ddagger} \longrightarrow \text{protein}^D\text{-}(DS^-)_{n,sat} \qquad (4b)$$

(iii) If denaturation is induced by interactions with pre-formed micelles of $C^+DS^-$ (Eq. 4c), the kinetics of denaturation should characterize the influence of $C^+$ bound to micelles of $DS^-$. In Eq. 4c, [protein-$(DS^-_{mic})_{\sim 60\text{-}100}$] is a structure involving the direct association of protein with a pre-formed micelle. We could not, however, predict whether $NR_4^+$ bound to the surface of micelles would increase rates of reaction (through favorable hydrophobic interactions of proteins with both $DS^-$ and $NR_4^+$) or decrease rates (by blocking the interaction of micelles of $DS^-$ with proteins).

$$\text{protein} + (DS^-_{mic})_{\sim 60\text{-}100} \longrightarrow [\text{protein-}(DS^-_{mic})_{\sim 60\text{-}100}]^{\ddagger} \longrightarrow \text{protein}^{D}\text{-}(DS^-)_{n,sat} \quad (4c)$$

**Testing Hypotheses by Analyzing the Denaturation of BCA.** The mechanism of denaturation of BCA in $Na^+DS^-$ is not known. Previous kinetic studies showed that the rate of denaturation increases by factors of ~$10^4$ with a small increase in the concentration of $Na^+DS^-$ (2.5 - 4.0 mM) just below the cmc (4.0 mM in Tris-Gly buffer).[25] Rates also increase with the concentration of $Na^+DS^-$ beyond the cmc, but only by a factor < 10 in the range of 4.0 - 10.0 mM $Na^+DS^-$. These data are ambiguous with regard to the mechanism of denaturation of BCA. The rates are sensitive to concentrations of $Na^+DS^-$ near the cmc and are therefore consistent with the idea that denaturation is induced by the assembly of $DS^-$ into micelles, templated by BCA (i.e., hypothesis (ii)). They do not however rule out hypothesis (i), which proposes that several equivalents of monomeric $DS^-$ interact with BCA to induce denaturation, but that these interactions are not related to micelle formation. By examining the effects of replacing

$Na^+$ with $NR_4^+$, and testing the hypotheses of Eq. 4a and 4b, we hoped to shed light on the mechanism of the reaction.

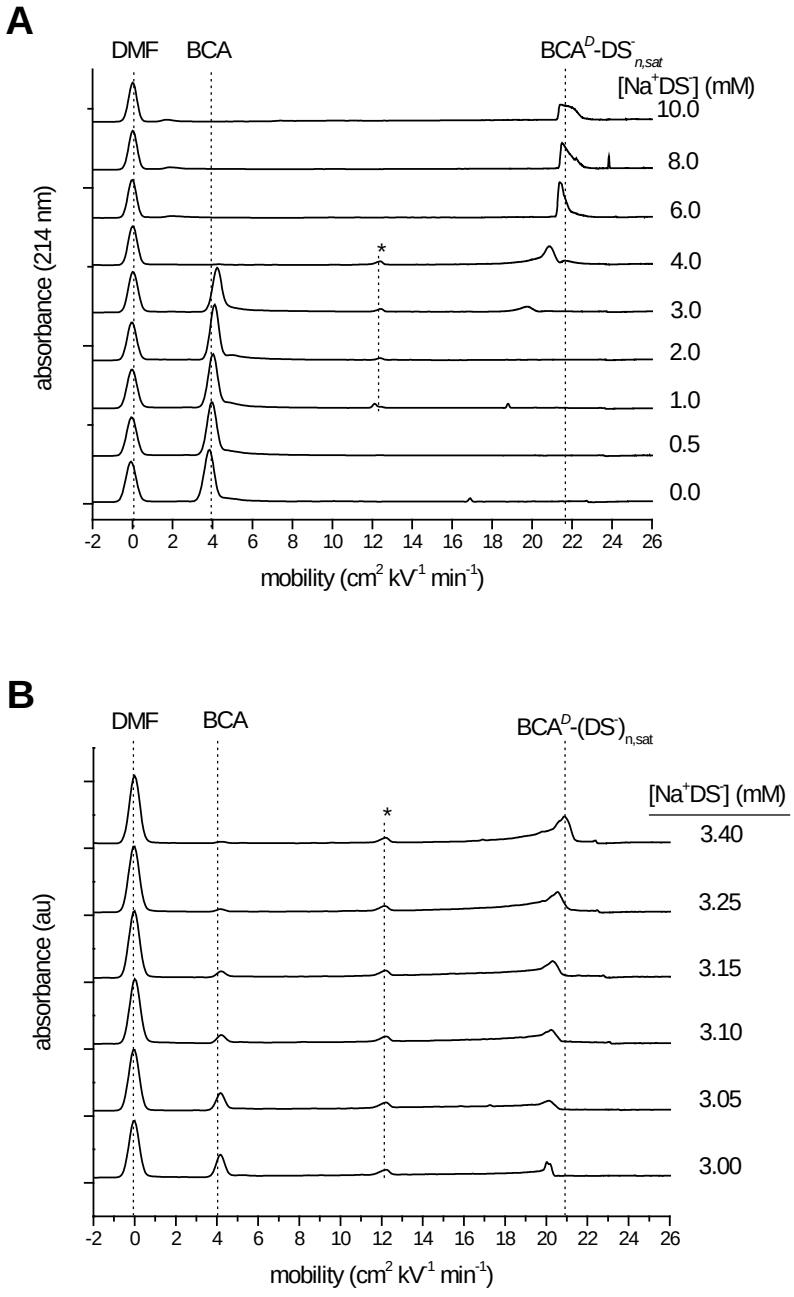
**Analysis by SurfCE: Formation of $BCA^D$-$DS^-_{n,sat}$ in Solutions of $Na^+DS^-$.** We used SurfCE to carry out and analyze reactions of BCA with $Na^+DS^-$. Traces obtained by injecting and analyzing samples of native BCA (8 μM) in capillaries with 0 - 10.0 mM $Na^+DS^-$ showed peaks for either native BCA or $BCA^D$-$DS^-_{n,sat}$ (Fig. 1A). SurfCE showed that the generation of $BCA^D$-$DS^-_{n,sat}$ is rapid at concentrations ≥ 6.0 mM; the reaction is complete within the interval of time of native BCA takes to reach the detector (~2.6 min.), under the conditions used to collect the data in Fig. 1A. The broadness of the peak in 4.0 mM $Na^+DS^-$ indicated that the time required for conversion to $BCA^D$-$DS^-_{n,sat}$ is 2 - 3 minutes at the cmc.

In the range of 3.00 - 3.40 mM $Na^+DS^-$, SurfCE data showed shoulders of peaks for $BCA^D$-$DS^-_{n,sat}$ (Fig. 1B); conversion of BCA to $BCA^D$-$DS^-_{n,sat}$ takes place over several minutes at these concentrations. Lower applied voltages (5 - 20 kV) in SurfCE provided longer windows of observation (retention times of native BCA were 4 - 16 min). Longer periods of time allowed for the complete conversion to $BCA^D$-$DS^-_{n,sat}$ in 3.00 mM $Na^+DS^-$ (data available in Fig. S3). Data obtained by SurfCE was therefore useful for examining reactions taking place over several minutes, and provided qualitative information about the rates of reactions (i.e., whether reactions take place over periods of time that are greater than, less than, or approximately the same as, the time required for the migration of native BCA in CE experiments).

Further discussion of the electropherograms in Fig. 1B is provided in the Supporting Information. Quantitative analysis of the absorbance over the broad range in

**Figure 1. Electropherograms obtained by SurfCE show the formation of $BCA^{D}$-$DS^{-}_{n,sat}$ in solutions of $Na^{+}DS^{-}$.** Samples of native BCA (8 μM) were injected into capillaries (60 cm in total length, 50 cm from the inlet to detector) filled with $Na^{+}DS^{-}$ in Tris-Gly buffer (25 mM Tris-192 mM glycine, pH = 8.4), and were analyzed by electrophoresis at 30 kV. Traces stacked in **(A)** cover the range 0.0 - 10.0 mM, and traces stacked in **(B)** focus on the range 3.00 - 3.40 mM. The small peak at ~12 $cm^2$ $kV^{-1}$ $min^{-1}$ (*) is not related to BCA. Fig. S4 in the Supporting Information identifies the peak as an artifact of stacking, caused by the injecting samples without $Na^{+}DS^{-}$ into capillaries containing running buffer with $Na^{+}DS^{-}$.

**Fig. 1**


A
DMF
BCA
BCA^D-DS^-_n,sat
[Na^+DS^-] (mM)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0
absorbance (214 nm)
mobility (cm^2 kV^-1 min^-1)
B
DMF
BCA
BCA^D-(DS^-)_n,sat
[Na^+DS^-] (mM)
3.40
3.25
3.15
3.10
3.05
3.00
absorbance (au)
mobility (cm^2 kV^-1 min^-1)

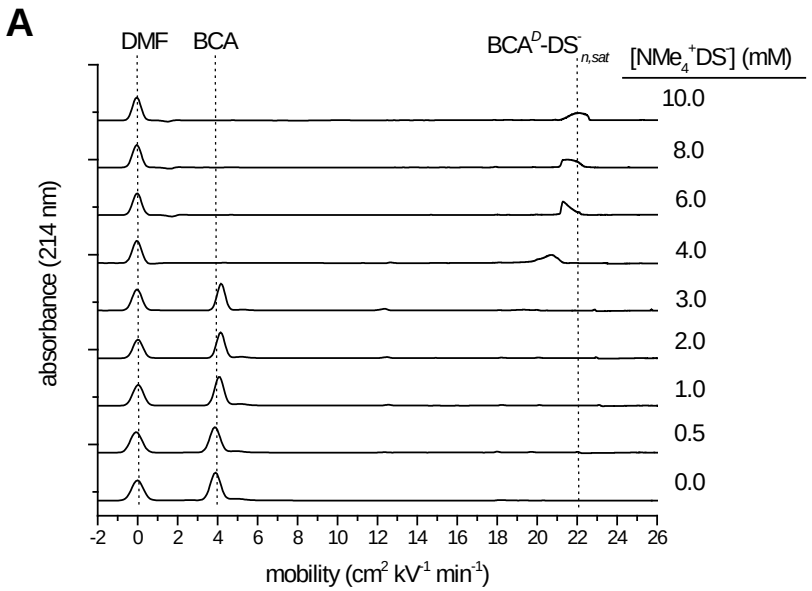
mobility ($4 < \mu < 22$ $cm^2$ $kV^{-1}$ $min^{-1}$) shows that the shapes of peaks are consistent with pseudo-first-order kinetics for the conversion of native BCA to $BCA^D$-$DS^-_{n,sat}$, during the SurfCE run (Figure S4). Rate constants determined by analyzing the peak shapes agreed with rate constants measured by an independent method (analysis of the fraction of BCA present in native form, measured using a fluorescent inhibitor of BCA, dansyl amide; Fig. S4).

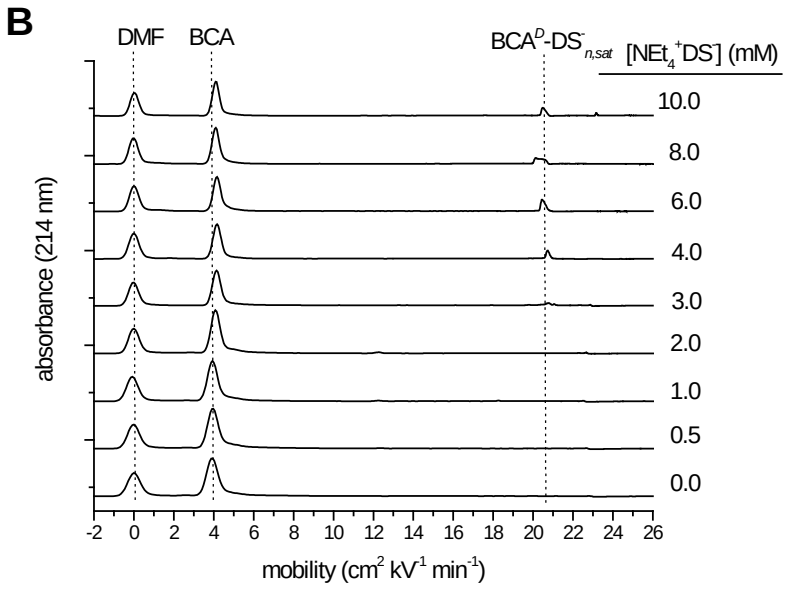
The formation of $BCA^D$-$DS^-_{n,sat}$ at concentrations of $Na^+DS^-$ below the cmc (4.0 mM) showed that interactions with monomeric $DS^-$ can lead to the formation of $BCA^D$-$DS^-_{n,sat}$; pre-formed micelles of $Na^+DS^-$ are not required for the denaturation of BCA. Analysis of BCA in solutions of $Na^+DS^-$ by SurfCE did not detect intermediates along the pathway to $BCA^D$-$DS^-_{n,sat}$.

**$NR_4^+$ decreases the rate of formation of $BCA^D$-$DS^-_{n,sat}$ in SurfCE.** SurfCE experiments analyzing BCA in solutions of $NR_4^+DS^-$ revealed that the structure of the cation influences the interaction of $DS^-$ with BCA. Figure 2 shows the analysis by SurfCE, of BCA in solutions of $NMe_4^+DS^-$ and $NEt_4^+DS^-$ (0 - 10 mM); data for $NPr_4^+DS^-$ and $Nu_4^+DS^-$ are in the Supporting Information. The amount of $BCA^D$-$DS^-_{n,sat}$ generated in 10.0 mM $NR_4^+DS^-$ decreased across the series, in the order $NMe_4^+$ (~ $Na^+$) > $NEt_4^+$ > $NPr_4^+$~ $NBu_4^+$. Solutions of $NEt_4^+DS^-$, even at concentrations well above the cmc, generated only small amounts of $BCA^D$-$DS^-_{n,sat}$ (< 20% conversion within the ~3 min. period of time allowed by SurfCE); only native BCA was observed in solutions 0 - 10 mM $NPr_4^+DS^-$ and $NBu_4^+DS^-$. The influence of $C^+$ in the SurfCE experiments was incompatible with the prediction of the hypothesis of Eq. 4b (i.e., that the formation of micelles of $DS^-$ would induce the formation of $BCA^D$-$DS^-_{n,sat}$). We

**Figure 2.** Samples of native BCA (8 μM) were analyzed by SurfCE in solutions of **(A)** $NMe_4^+DS^-$ and **(B)** $NEt_4^+DS^-$, at concentrations of 0 - 10 mM. SurfCE experiments used an applied voltage of 30 kV and capillaries that were 60 cm in length, with 50 cm between inlet and detector. *N*,*N*-dimethylformamide (DMF, 2 mM) was added to samples as an internal standard for the purpose of indicating electro-osmotic flow. In **(B)**, the small peaks near $\mu$ = 21 $cm^2\ kV^{-1}\ min^{-1}$ are probably $BCA^D$-$DS^-_{n,sat}$; other experiments allowing longer intervals of time (i.e., CZE) show that the peak for native BCA disappears as the peak near 21 $cm^2\ kV^{-1}\ min^{-1}$ increases in area.

**Fig. 2 (A)**


A
DMF
BCA
BCA^D-DS^-_n,sat
[NMe4+DS-] (mM)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0
absorbance (214 nm)
-2 0 2 4 6 8 10 12 14 16 18 20 22 24 26
mobility (cm2 kV-1 min-1)

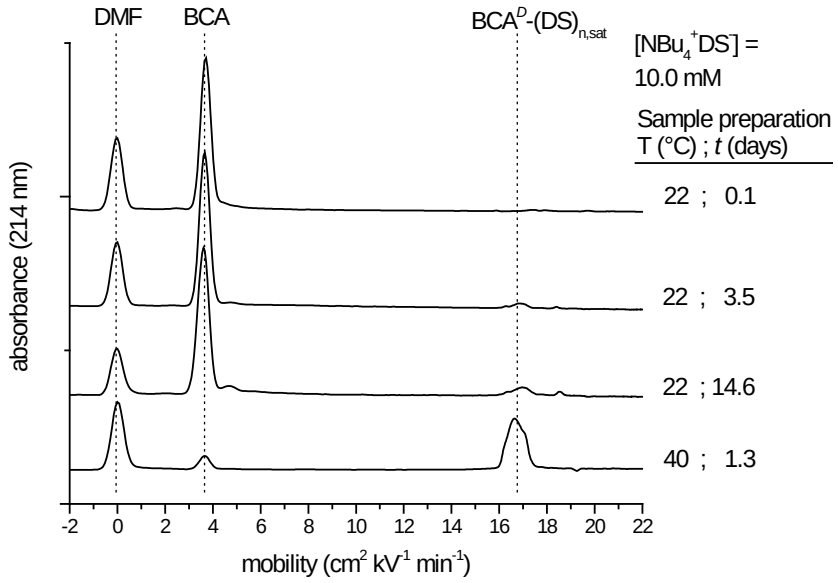

B
DMF
BCA
BCA^D-DS^-_n,sat
[NEt4+DS-] (mM)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0
absorbance (214 nm)
-2 0 2 4 6 8 10 12 14 16 18 20 22 24 26
mobility (cm2 kV-1 min-1)

concluded that the formation of $BCA^{D}$-$DS^{-}_{n,sat}$ in solutions of $NR_4^+DS^-$ is not related to the formation of micelles of $NR_4^+DS^-$. The data however remain compatible with the prediction of Eq. 4a: substitution of $NR_4^+$ for $Na^+$ results in decreasing amounts of $BCA^{D}$-$DS^{-}_{n,sat}$ because the denaturation of BCA is induced by monomeric $DS^-$. We hypothesized that the formation of lower amounts of $BCA^{D}$-$DS^{-}_{n,sat}$ in $NR_4^+DS^-$ with increasingly hydrophobic $NR_4^+$ was the result of decreasing concentrations of monomeric $DS^-$, due to micelle formation at lower values of cmc.

SurfCE data in Fig. 2 however provided a window of observation limited to reactions of BCA with $DS^-$ that take place within 2 - 3 minutes. From these experiments, the underlying reasons for the absence of $BCA^{D}$-$DS^{-}_{n,sat}$ in solutions of $NPr_4^+DS^-$ and $NBu_4^+DS^-$ are unclear. Other experiments – observation over longer periods of time, or acceleration of reactions by heating – were necessary to determine whether the SurfCE results were outcomes of thermodynamics favoring native BCA, or slow kinetics of denaturation.

**Generation of $BCA^{D}$-$DS^{-}_{n,sat}$ in $NBu_4^+DS^-$.** BCA remained folded in solutions of 10 mM $NBu_4^+DS^-$, kept at 22 °C, for several weeks. After 3.5 and 15 days of incubation, analysis by capillary zone electrophoresis (CZE) showed that BCA in the sample was in its native form (Fig. 3) (analysis of samples after 100 days showed a decrease in total absorbance; we did not determine the reason for the apparent loss in total protein). We identified the small peak that developed at $\mu \sim 17$ $cm^2$ $kV^{-1}$ $min^{-1}$ as $BCA^{D}$-$DS^{-}_{n,sat}$, by showing that it has the same mobility as the major peak for samples heated to 40 °C (for 32 hours). These results showed that the formation of $BCA^{D}$-$DS^{-}_{n,sat}$ in $NBu_4^+DS^-$ is slow, and that CZE data showing native BCA in 10 mM $NBu_4^+DS^-$ are early snapshots of

**Figure 3. Denaturation of BCA in 10.0 mM $NBu_4^+DS^-$ at 22 ˚C.** BCA in 10.0 mM $NBu_4^+DS^-$, kept at 22 ˚C, was analyzed at various time points by CZE (times given to the right of the trace). CZE experiments used an applied voltage of 30 kV and a capillary that was 60 cm in length, with 50 cm between the inlet and detector. The bottom trace is for reference; a sample of $BCA^D$-$DS^-_{n,sat}$ was generated by heating a solution of BCA in 10.0 mM $NBu_4^+DS^-$ to 40 ˚C.



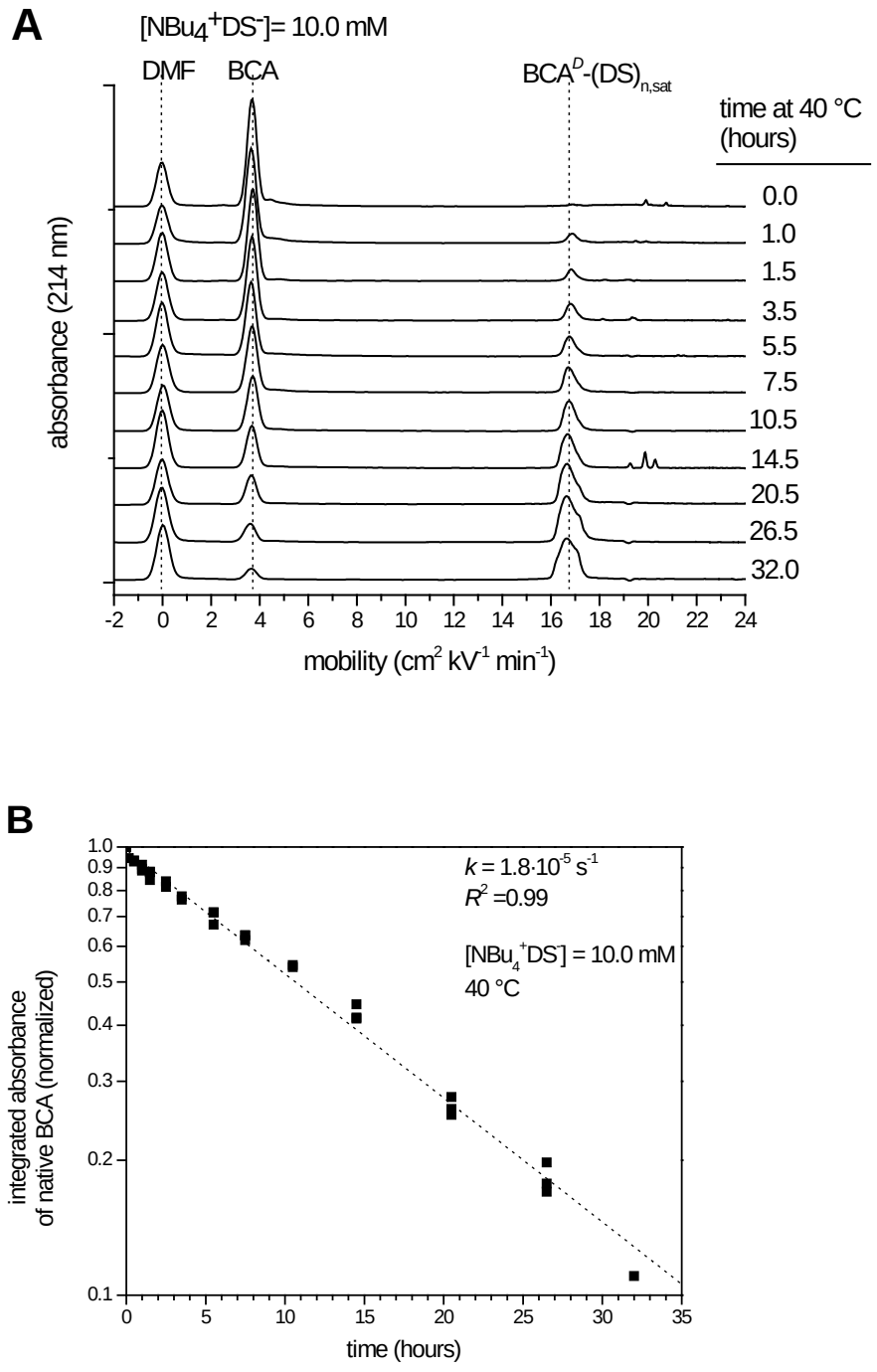
a slow reaction at 22 °C (half-life greater than 10 days). The formation of $BCA^{D}$-$DS^{-}_{n,sat}$ at 22 °C is slower in 10.0 mM $NBu_4^+DS^-$ than in 10.0 mM $Na^+DS^-$ by a factor > $10^4$ (the approximation is based on reactions that require an amount of time < 1 min. in 10.0 mM $Na^+DS^-$ but > 10 days in 10.0 mM $NBu_4^+DS^-$). We inferred that the results of SurfCE in Fig. 2 were due to the kinetics of the system: decreasing amounts of $BCA^{D}$-$DS^{-}_{n,sat}$ in SurfCE are the result of a decrease in the rates of reaction between $DS^-$ and BCA in solutions containing $Na^+$ or $NR_4^+$.

**Kinetics of Formation of $BCA^{D}$-$DS^{-}_{n,sat}$ at 40 °C.** To circumvent the sluggish kinetics in solutions of $NBu_4^+DS^-$ at 22 °C, we analyzed the rate of formation of $BCA^{D}$-$DS^{-}_{n,sat}$ at 40 °C (a temperature still substantially below the melting temperature of BCA, which is 64 °C)[84]. Traces collected by CZE, at times up to 32 hours, showed the conversion of BCA to $BCA^{D}$-$DS^{-}_{n,sat}$ in solutions of 10 mM $NBu_4^+DS^-$ (Fig. 4A). Areas of peaks for native BCA and $BCA^{D}$-$DS^{-}_{n,sat}$ allowed us to quantify the amount of native BCA and to estimate a value of $1.8 \cdot 10^{-5}$ $s^{-1}$ for the pseudo-first-order rate constant for the disappearance of native BCA (Fig. 4B). We ignored the decrease in the concentration of $NBu_4^+DS^-$ expected over the course of the reaction, caused by the absorption of ~150 equivalents of $DS^-$ by BCA.[94] The rate of formation of $BCA^{D}$-$DS^{-}_{n,sat}$ in 10.0 mM $NPr_4^+DS^-$ at 40 °C ($2.1 \cdot 10^{-4}$ $s^{-1}$) was greater than that in 10.0 mM $NBu_4^+DS^-$ by a factor of ~10 (Figure S6).

The formation of $BCA^{D}$-$DS^{-}_{n,sat}$ at 40 °C in solutions of 10.0 mM $Na^+DS^-$, $NMe_4^+DS^-$, and $NEt_4^+DS^-$ was too rapid for analysis by CZE. Instead of using CZE, we used a spectroscopic method to measure the rates of denaturation of BCA at these conditions. We analyzed the rates of denaturation of BCA by measuring the fluorescence

**Figure 4. Denaturation of BCA in 10.0 mM $NBu_4^+DS^-$ at 40 °C. (A)** CZE traces analyzing aliquots taken from mixtures of BCA (10 μM) and 10.0 mM $NBu_4^+DS^-$ heated to 40 °C. At times noted, aliquots were injected into capillaries (60 cm in length, 50 cm between the inlet and detector) filled with 10.0 mM $NBu_4^+DS^-$ in Tris-Gly buffer (22 °C), and analyzed by electrophoresis at 30 kV. **(B)** Points represent the fraction of BCA that was in native form, determined from areas of peaks for native BCA and $BCA^D$-$DS^-_{n,sat}$, (corrected for differences in the residence time at the UV-detector). All points from three trials are included. Analysis by single-exponential decay (dotted line) provided an estimate for the first-order rate constant.

**Fig. 4**


A
[NBu4+DS-]= 10.0 mM
DMF
BCA
BCAD-(DS)n,sat
time at 40 °C
(hours)
0.0
1.0
1.5
3.5
5.5
7.5
10.5
14.5
20.5
26.5
32.0
absorbance (214 nm)
-2 0 2 4 6 8 10 12 14 16 18 20 22 24
mobility (cm2 kV-1 min-1)
B
k = 1.8·10-5 s-1
R2 =0.99
[NBu4+DS-] = 10.0 mM
40 °C
integrated absorbance
of native BCA (normalized)
1.0 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1
0 5 10 15 20 25 30 35
time (hours)

intensity of dansyl amide (DNSA) bound to the active site of folded BCA; the fluorescence of DNSA at 460 nm increases by a factor >100 upon binding the active site of BCA.[25, 95, 96] By adding DNSA to aliquots taken from reaction mixtures of BCA and $C^+DS^-$ at points over time, and measuring the fluorescence of DNSA, we quantified the amount of BCA that remained folded and determined the rate of denaturation. Figure 5 shows rate constants for the denaturation of BCA in solutions of $Na^+DS^-$ and $NR_4^+DS^-$ at 40 °C. (Examples of raw data for DNSA fluorescence are available in Fig. S7).

Rates of denaturation spanned factors of ~$10^4$ in solutions of 10.0 mM $C^+DS^-$ at 40 °C, and decreased in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. Denaturations in solutions of either $Na^+DS^-$, $NMe_4^+DS^-$, or $NEt_4^+DS^-$ were rapid and almost complete within the time required to mix BCA with solutions of surfactant manually (< 4 s; points in Fig. 5 overlap).

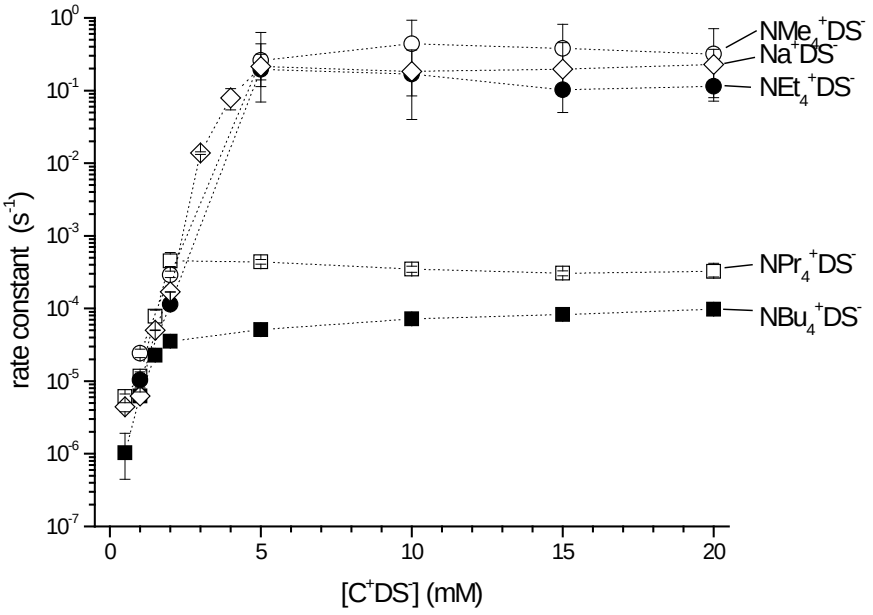
The data in Fig. 5 reveal three key features in the kinetics of denaturation of BCA: (i) Rates are similar for different $C^+DS^-$ when the concentration of $C^+DS^-$ is below the cmc. (ii) The increase in rate essentially stops at the cmc (rates increase by factors ≤ 10 as $[C^+DS^-]$ rises just beyond the cmc, and are indistinguishable at $[C^+DS^-]$ well above the cmc).[97] (iii) the increase in rate constant is not a simple function of the concentration of $C^+DS^-$. Below the cmc, the relationship between the rate of denaturation and the concentration of $DS^-$ is approximately given by Eq. 5a and 5b.

$$k_{den} \alpha \ 10^{[DS^-]} \qquad (5a)$$

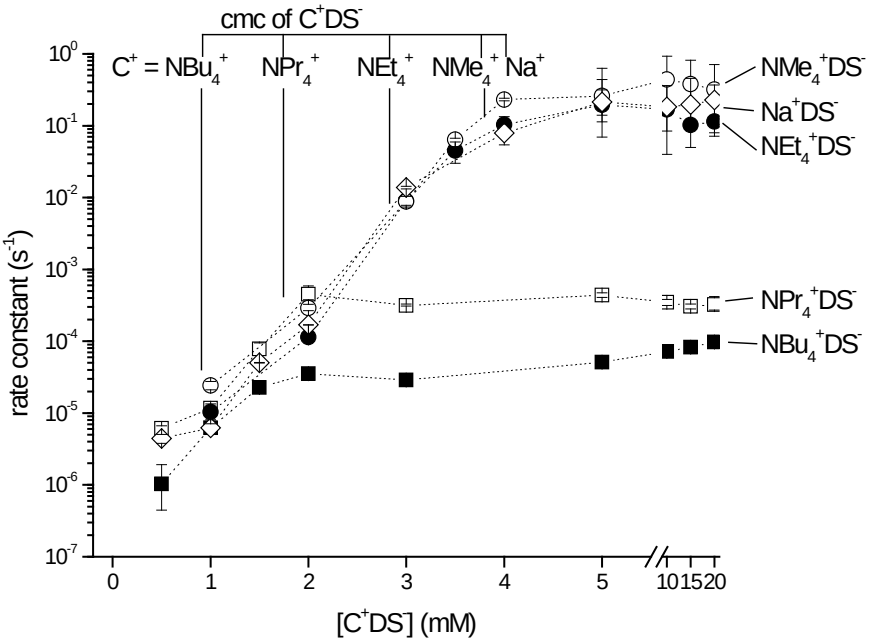
$$\log k_{den} \alpha \ \Delta G^{\mp} \alpha [DS^-] \qquad (5b)$$

**Figure 5. Rate Constants for the Denaturation of BCA in Solutions of $NR_4^+DS^-$ at 40 °C.** Points show rate constants that were measured by analyzing aliquots taken from mixtures of BCA (0.2 μM) and $C^+DS^-$ in Tris-Gly buffer heated to 40 °C. Aliquots were analyzed by adding DNSA, and measuring the fluorescence intensity at 460 nm ($\lambda_{ex}$ =280 nm; concentrations of DNSA and BCA were 2 μM and 0.1 μM respectively). Rate constants were estimated by fitting a single-exponential decay to the decrease in fluorescence intensity over time. For rapid reactions (rate constants of $10^{-1}$ - $10^{0}$ $s^{-1}$), samples were analyzed ~4 - 8 s after mixing $C^+DS^-$ and BCA. For "slow" reactions (rate constants < $10^{-3}$ $s^{-1}$), intervals of time were 0.5 - 4.0 hrs. Points are averages of three or four repetitions, and error bars span minimum and maximum values. Straight, dotted lines that connect the points are to guide the eye. Inset: Magnification of data at 0 - 5.0 mM $NR_4^+DS^-$. We did not observe any change in the amount of native BCA in solutions of any surfactant at concentrations of 0.25 mM after 48 hours; we did not analyze these samples at longer periods of time.

**Figure 5.**




**Inset:**



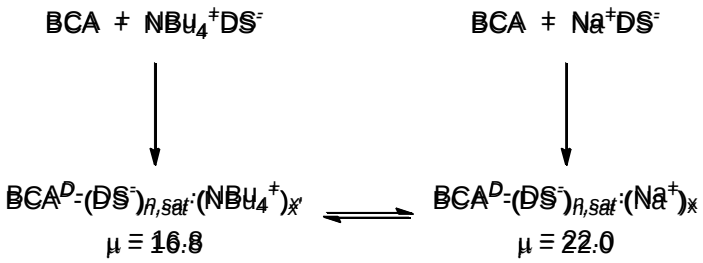
In Eq. 5, $k_{den}$ is the rate constant for denaturation (with units $s^{-1}$), and [$DS^-$] (mM) is the concentration of $C^+DS^-$ below the cmc.

According to this interpretation of Fig. 5, $NR_4^+$ affects the reaction of $DS^-$ with BCA by setting an upper limit on the concentration of monomeric $DS^-$. In any solution of $C^+DS^-$, the formation of micelles at the cmc limits the concentration of monomeric $DS^-$ that can be reached in the presence of $C^+$. We conclude that the rate of denaturation is determined by the concentration of monomeric $DS^-$, and not by micelles of $C^+DS^-$. The kinetics indicate a rate-limiting step that involves monomeric $DS^-$. Values for the rate constant $k_{den}$ do not have a linear dependence on [$DS^-$]; the rate-limiting step therefore involves the interaction of BCA with many equivalents of $DS^-$ (we do not know how many equivalents of $DS^-$ from these data). Although interactions of monomeric $DS^-$ with BCA may be cooperative, the data of Fig. 5 suggest that these interactions are not the same as those involved in the self-association of monomeric $DS^-$ to form micelles of $DS^-$. Instead, the initial step for denaturation may be the association of monomeric $DS^-$ (possibly several equivalents) with native BCA that induces local unfolding and exposure of hydrophobic surface area; subsequent association of more monomeric $DS^-$ to partially unfolded protein may cause the denaturation of BCA and formation of $BCA^D$-$DS^-_{n,sat}$. Since we do not observe shifts in $\mu$ in SurfCE to suggest stable complexes of BCA with a small number of equivalents of $DS^-$, we conclude that the unfolding of BCA is highly cooperative and rapid when the concentration of $C^+DS^-$ reaches an appropriate value.

**Values of mobility for $BCA^D$-$DS^-_{n,sat}$ suggest that $NBu_4^+$ and $Na^+$ associate with $BCA^D$-$DS^-_{n,sat}$.** Values of $\mu$ for $BCA^D$-$DS^-_{n,sat}$ were lower in 10.0 mM $NBu_4^+DS^-$

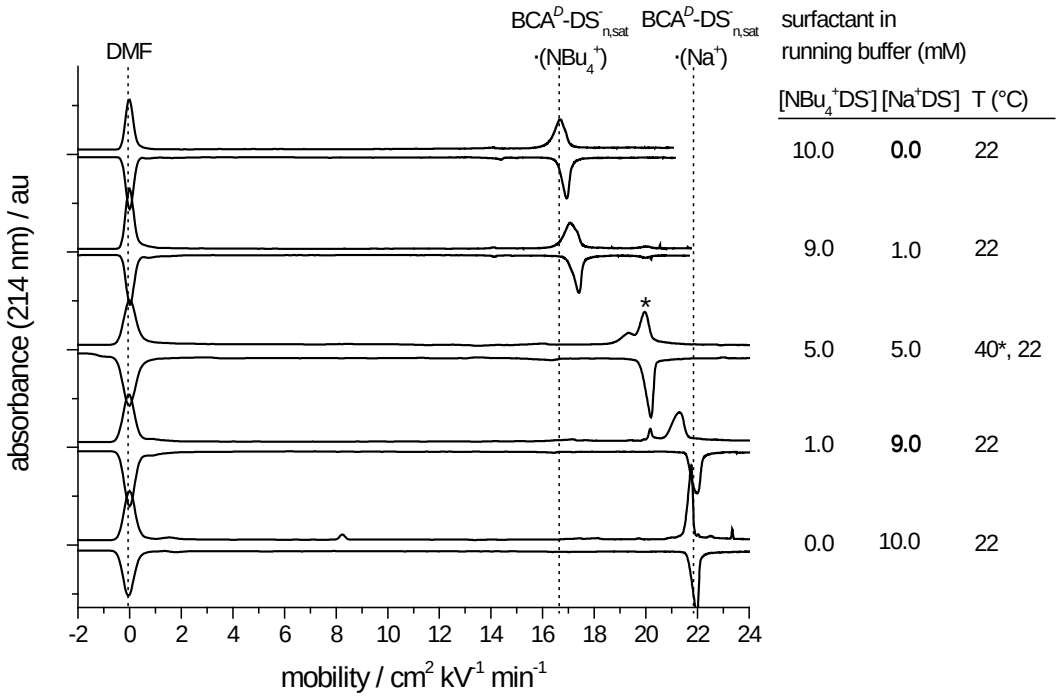
(16.8 $cm^2\ kV^{-1}\ min^{-1}$) than in 10.0 mM $Na^+DS^-$ (22.0 $cm^2\ kV^{-1}\ min^{-1}$) (Figure 6). The shift in $\mu$ suggested the influence of the cation on the composition of $BCA^D$-$DS^-_{n,sat}$. To demonstrate that the mobility of $BCA^D$-$DS^-_{n,sat}$ depends on the cation ($Na^+$ or $NBu_4^+$) in solution, but does not depend on how $BCA^D$-$DS^-_{n,sat}$ is prepared, we carried the reactions in Scheme 1.

**Scheme 1:**




We generated two samples of $BCA^D$-$DS^-_{n,sat}$ – one in $NBu_4^+DS^-$ and another in $Na^+DS^-$ – by heating BCA (8 μM) in solutions of 10.0 mM $NBu_4^+DS^-$ or 10.0 mM $Na^+DS^-$ (40 °C, 56 hours). We analyzed $BCA^D$-$DS^-_{n,sat}$ from both preparations, after treating samples to a three-step procedure: (i) concentration of the protein to ~80 μM, using a membrane with a 10-kDa molecular weight cutoff; (ii) ten-fold dilution into 10.0 mM $NBu_4^+DS^-$ (and in parallel, ten-fold dilution into 10.0 mM $Na^+DS^-$); (iii) incubation for 20 min. at 22 °C. Both preparations of $BCA^D$-$DS^-_{n,sat}$, after equilibration in 10.0 mM $NBu_4^+DS^-$, gave CZE traces showing agreement in $\mu$ (~ 16.8 $cm^2\ kV^{-1}\ min^{-1}$, top set of traces in Fig. 6; running buffer contained 10.0 mM $NBu_4^+DS^-$). CZE traces also agreed for $BCA^D$-$DS^-_{n,sat}$ that had been equilibrated in 10.0 mM $Na^+DS^-$ ($\mu$ ~ 22.0 $cm^2\ kV^{-1}\ min^{-1}$, bottom set of traces in Fig. 6).

**Figure 6. Analysis of Equilibrated Samples of $BCA^{D}$-$DS^{-}_{n,sat}$ by CZE.** Samples of $BCA^{D}$-$DS^{-}_{n,sat}$, prepared by heating BCA in either 10.0 mM $NBu_4^+DS^-$ or 10.0 mM $Na^+DS^-$ for 56 hours, were diluted ten-fold into solutions of 10.0 mM $DS^-$ and mixtures of $NBu_4^+$ and $Na^+$. After equilibration for twenty minutes at 22 °C, samples were analyzed by CZE. Traces are shown for the analysis of $BCA^{D}$-$DS^{-}_{n,sat}$ prepared initially in $NBu_4^+DS^-$ (peaks up) or in $Na^+DS^-$ (peaks down). Samples analyzed in $[NBu_4^+]$ = $[Na^+]$ = 5.0 mM (marked by *) were equilibrated at 40 °C for 6 hrs.



We drew three conclusions from the results: (i) Conversion between $BCA^{D}$-$DS^{-}_{n,sat}$ with $\mu$ = 16.8 and $BCA^{D}$-$DS^{-}_{n,sat}$ with $\mu$ = 22.0 is reversible at 22 °C (t ~ 20 min; intervals of time allowed by SurfCE were not sufficient to equilibrate the sample); (ii) The value of $\mu$ for $BCA^{D}$-$DS^{-}_{n,sat}$ depends on the cation, suggesting the $Na^{+}$ and $NBu_4^{+}$ associate with $BCA^{D}$-$DS^{-}_{n,sat}$, and that the complexes should be viewed as $BCA^{D}$-$DS^{-}_{n,sat}\cdot(C^{+})$; iii) The influence of $Na^{+}$ and $NBu_4^{+}$ on the mobility of $BCA^{D}$-$DS^{-}_{n,sat}$ is determined by association to $BCA^{D}$-$DS^{-}_{n,sat}$ and is independent of how $BCA^{D}$-$DS^{-}_{n,sat}$ is formed.

Analysis of the data in Figure 6 to estimate the stoichiometry and affinity of $NBu_4^{+}$ and $Na^{+}$ for $BCA^{D}$-$DS^{-}_{n,sat}$ is included in the Supporting Information (Fig. S8).

**Other Proteins.** We wished to determine whether the influence of $NR_4^{+}$ on the formation of aggregates of protein and $DS^{-}$, protein-$DS^{-}_{n}$, was similar for BCA and other proteins. We used SurfCE to survey seven additional proteins (listed in Table 2) in solutions of 0 - 10.0 mM $Na^{+}DS^{-}$ and $NR_4^{+}DS^{-}$. Figure 7 shows SurfCE traces for these proteins in solutions of $Na^{+}DS^{-}$ and of $NBu_4^{+}DS^{-}$; data for analysis in $NMe_4^{+}DS^{-}$, $NEt_4^{+}DS^{-}$, and $NPr_4^{+}DS^{-}$ are available in Fig. S9.

The results of SurfCE revealed a range of behaviors among the proteins tested. For several proteins (β-LacB, α-Lac, MYO, UBI), SurfCE data collected in $NR_4^{+}DS^{-}$ and $Na^{+}DS^{-}$ was similar, suggesting that the influence of the cation on the formation of protein-$DS^{-}_{n}$ was relatively minor. For others (BCA, CPK,[98] and β-LacA), replacing $Na^{+}$ with $NBu_4^{+}$ decreased the amount of protein-$DS^{-}_{n}$ generated in SurfCE. For CPB, the effect of the cation was distinctive; replacing $Na^{+}$ with $NPr_4^{+}$ or $NBu_4^{+}$ enabled the formation of complexes of CPB with $DS^{-}$ that are not observed in solutions of $Na^{+}DS^{-}$.

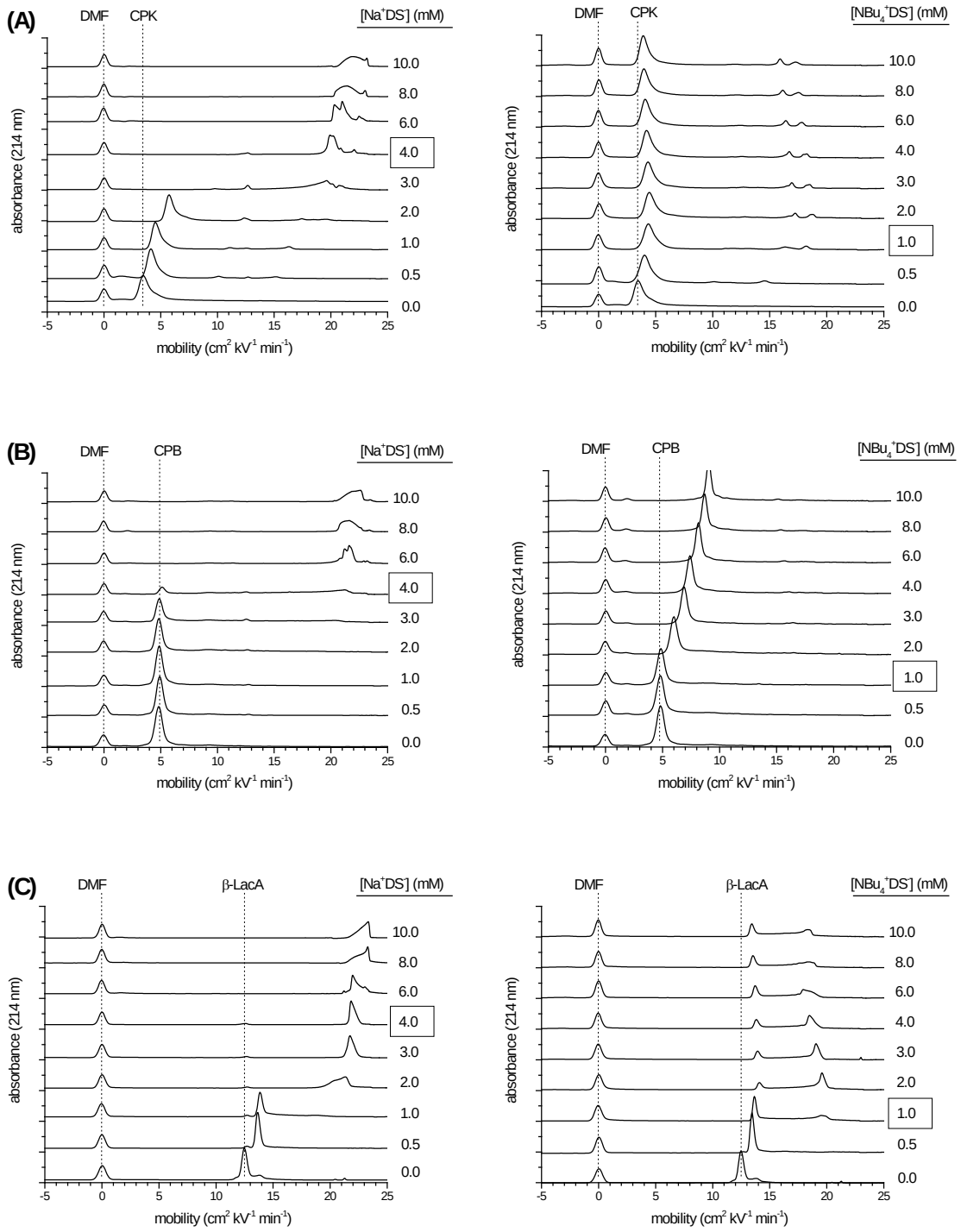
**Table 2.** Proteins Analyzed by SurfCE in Solutions of $Na^+DS^-$ and $NR_4^+DS^-$

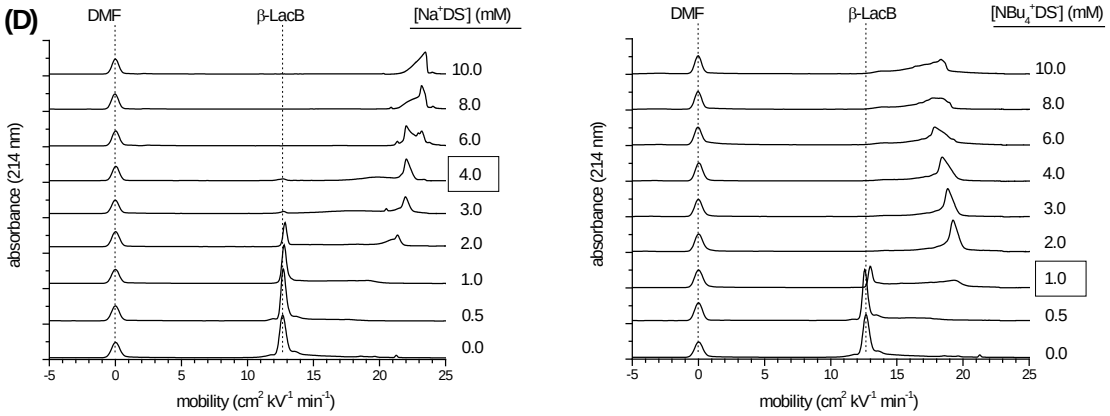
| protein | enzyme accession no.[a] | molecular weight[a] | p*I*[a] | [$Na^+DS^-$] for $\Delta\mu$[b] | $\Delta\mu_{Na^+,NBu_4^+}$[c] |
|---|---|---|---|---|---|
| bovine carbonic anhdrase (BCA) | P00921 | 30.0 | 5.9 | 3.0 | 18.0 |
| creatine phosphokinase (CPK)[d] | P005663 | 86.2 | 6.7 | 3.0 | 18.5 |
| carboxypeptidase B (CPB) | P09955 | 34.7 | 5.7 | 4.0 | 12.8 |
| β-lactoglobulin A (β-LacA) | P02754 | 18.4 | 4.8 | 2.0 | 5.0 |
| β-lactoglobulin B (β-LacB) | P02754 | 18.3 | 4.8 | 2.0 | 4.0 |
| α-lactalbumin (α-Lac) | P00711 | 14.2 | 4.8 | 1.0 | 4.5 |
| myoglobin (MYO) | P68082 | 17.0 | 7.4 | 0.5 | 4.5 |
| ubiquitin (UBI) | P62990 | 8.6 | 6.6 | 1.0 | 2.0 |

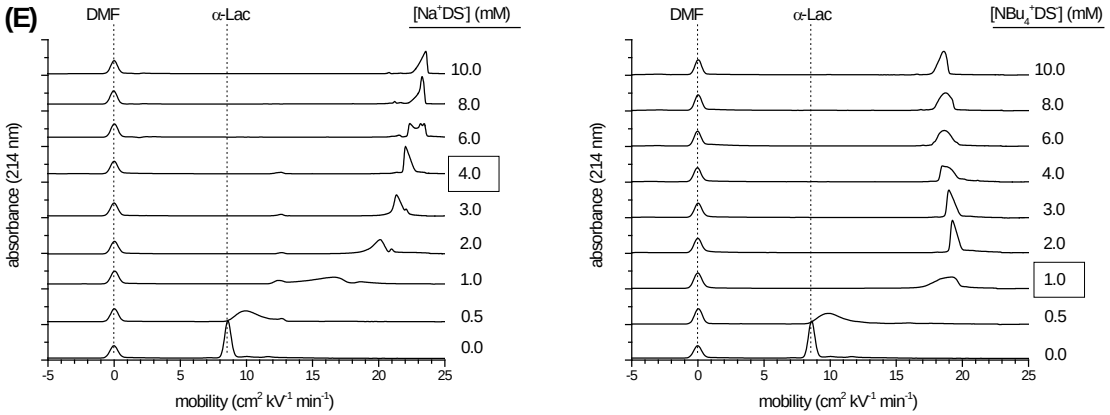
[a] Data for the molecular weight (kDa) and p*I* were obtained from the Swiss-Prot database. [b] Concentrations of $Na^+DS^-$ that cause shifts in mobility > 5 $cm^2\ kV^{-1}\ min^{-1}$ in SurfCE experiments, determined from the data in Fig. 9 (SurfCE experiments observed reactions taking place within 2 - 3 min.). [c] Difference between the mobility of the major peaks observed in SurfCE (reactions taking place within 2 - 3 min.) using 10 mM $Na^+DS^-$ and 10 mM $NBu_4^+DS^-$. [d] CPK is a homo-dimer and contains no disulfide bonds.

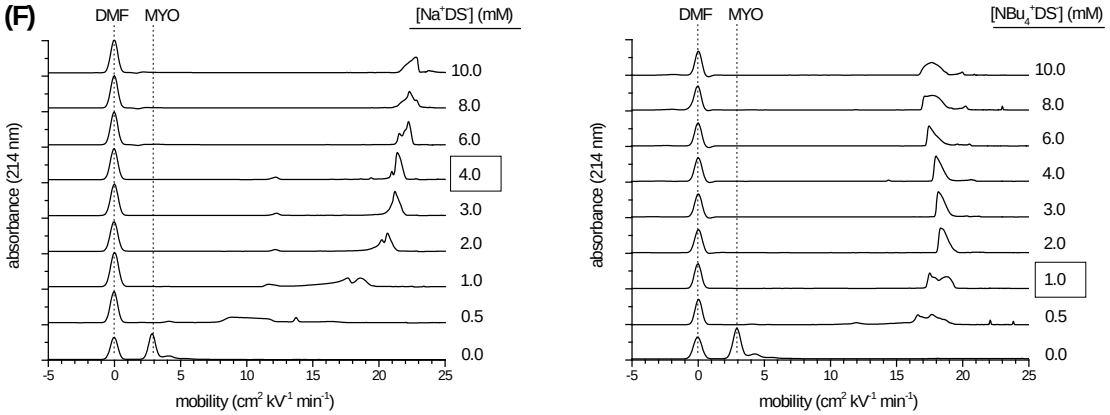
**Figure 7.** SurfCE data for the analysis of **(A)** CPK, **(B)** CPB, **(C)** β-LacA, **(D)** β-LacB, **(E)** α-Lac, **(F)** MYO, **(G)** UBI in solutions of $Na^+DS^-$ or $NBu_4^+DS^-$. Samples of protein (~0.3 mg/mL) in Tris-Gly were injected into capillaries filled with solutions of surfactant, and analyzed by electrophoresis (30 kV using capillaries that were 60 cm in length and 50 cm between inlet and detector). In the data for CPK in $NBu_4^+DS^-$, small peaks at $\mu$ ~ 17 and 19 $cm^2\ kV^{-1}\ min^{-1}$ have not been identified. Data for analysis in solutions of $NMe_4^+DS^-$, $NEt_4^+DS^-$, and $NPr_4^+DS^-$ are available in Fig. S9.

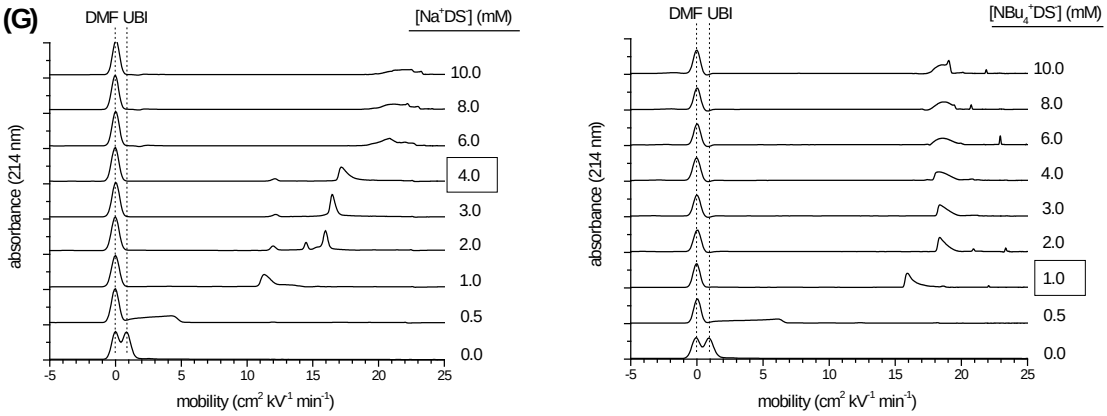
**Fig. 7**


(A)
DMF
CPK
[Na+DS-] (mM)
[NBu4+DS-] (mM)
absorbance (214 nm)
mobility (cm2 kV-1 min-1)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0
(B)
CPB
(C)
β-LacA


(D)
DMF
β-LacB
[Na+DS-] (mM)
[NBu4+DS-] (mM)
absorbance (214 nm)
mobility (cm2 kV-1 min-1)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0


(E)
DMF
α-Lac
[Na+DS-] (mM)
[NBu4+DS-] (mM)
absorbance (214 nm)
mobility (cm2 kV-1 min-1)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0


(F)
DMF
MYO
[Na+DS-] (mM)
[NBu4+DS-] (mM)
absorbance (214 nm)
mobility (cm2 kV-1 min-1)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0


(G)
DMF UBI
[Na+DS-] (mM)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0
absorbance (214 nm)
-5
0
5
10
15
20
25
mobility (cm2 kV-1 min-1)
DMF UBI
[NBu4+DS-] (mM)
10.0
8.0
6.0
4.0
3.0
2.0
1.0
0.5
0.0
absorbance (214 nm)
-5
0
5
10
15
20
25
mobility (cm2 kV-1 min-1)

The influence of $NR_4^+$ appeared to distinguish these proteins by their mechanism of denaturation. Proteins that showed only minor changes upon replacement of $Na^+$ with $NR_4^+$ in SurfCE were also those that denatured in solutions of $Na^+DS^-$ at concentrations (1 - 2 mM) well below the cmc (i.e., β-LacB, α-Lac, MYO, UBI). Denaturation of these proteins may be induced by interactions with monomeric $DS^-$ at low concentration; we would not expect the influence of $NR_4^+$ on the cmc or on the properties of micelles of $NR_4^+DS^-$ to affect the denaturation of these proteins.

Proteins that did not denature in $NPr_4^+DS^-$ and $NBu_4^+DS^-$ were also those that require concentrations of $Na^+DS^-$ (3 - 4 mM) near the cmc for denaturation (BCA and CPK). These concentrations of $Na^+DS^-$ may be important for denaturation because the affinity of these proteins for $DS^-$ may be low. As was the case for BCA, the influence of $NR_4^+$ on the denaturation of CPK may be due to concentrations of monomeric $DS^-$ that are limited by the formation of micelles of $NR_4^+DS^-$ at decreasing values of cmc. The formation of CPB-$DS^-_n$ in $NBu_4^+DS^-$ with mobilities of 6 - 10 $cm^2\ kV^{-1}\ min^{-1}$ suggested a different type of involvement of $NBu_4^+$; we do not understand the reason for the gradual shift in $\mu$ for this protein, other than it seems to involve association of $DS^-$ without complete denaturation.[99]

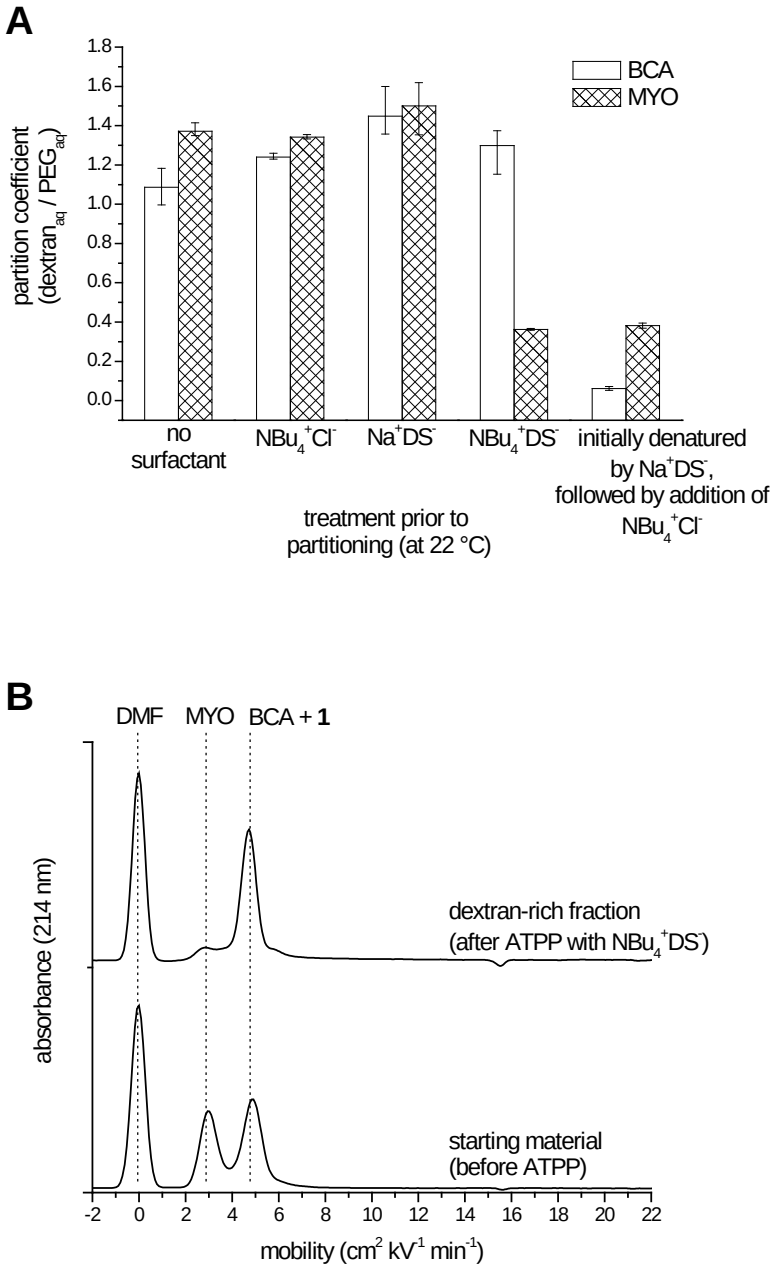
**Application of $NBu_4^+DS^-$ in Methods for Separating Proteins.** In principle, the selective and differential formation of protein-$DS^-_n$ from proteins in a mixture should be useful in the analysis or purification of proteins. Gudiksen demonstrated the resolution of proteins in CE in solutions of $Na^+DS^-$, below the cmc, by exploiting differences in the rates of denaturation.[38] The combination of Aqueous Two-Phase Partitioning (ATPP) and the selective formation of protein$^D$-$DS^-_{n,sat}$ in $NBu_4^+DS^-$ should also be useful for

separating proteins. Most applications of ATPP in protein purification rely on differences in partition coefficient in mixtures of aqueous dextran and poly(ethylene glycol) (PEG); the dextran-rich phase is considered more hydrophilic than the PEG-rich phase.[100-102,100, 102-104] We hypothesized that ATPP would be able to separate native proteins from protein$^{D}$-$DS^{-}_{n,sat}$.

To show how the treatment of proteins with $NBu_4^+DS^-$ can enhance separations by ATPP, we analyzed the partitioning of MYO (a protein that denatures in $NBu_4^+DS^-$ at 22 °C in SurfCE experiments) and BCA (a protein that is kinetically stable towards denaturation in $NBu_4^+DS^-$ at 22 °C). We measured the partition coefficients for samples of MYO and BCA prepared by adding these proteins to five different solutions (all in Tris-Gly buffer at 22 °C): (i) buffer only; (ii) 16.7 mM $NBu_4^+Cl^-$; (iii) 16.7 mM $Na^+DS^-$; (iv) 16.7 mM $NBu_4^+DS^-$; (v) 20.0 mM $Na^+DS^-$ for five minutes, followed by the addition of $NBu_4^+Cl^-$, resulting in a concentration of 16.7 mM for $DS^-$, $Na^+$, $NBu_4^+$, and $Cl^-$. The procedure in (v) was designed to obtain BCA$^{D}$-$DS^{-}_{n,sat}$ and MYO $^{D}$-$DS^{-}_{n,sat}$ in solutions containing $DS^-$ and $NBu_4^+$, without heating. We added 360 μLs of the samples in (i) - (v) to 300 μLs of a stock mixture consisting of 8% PEG and 12% dextran (w/vol) in Tris-Gly buffer. Final concentrations of PEG and dextran in the partitioning mixtures were 3.6% and 5.5% respectively; the concentration of $DS^-$, for partitioning samples (iii) - (v), was 9.1 mM (at this concentration of surfactant, neither denatured BCA or denatured MYO refold). After vortexing the mixture for 10 s and allowing it to equilibrate for 20 min (without further agitation), we separated the PEG-rich and dextran-rich phases by centrifugation and determined the partition coefficients by measuring the absorbance of BCA and MYO (280 nm) in each phase (data in Fig. 8).

**Figure 8. Aqueous Two-phase Partitioning of BCA and Myoglobin (MYO). (A)** Partition coefficients for BCA or MYO that had been treated with solutions (i) - (v) described in the text. Samples of protein (0.3 mg/mL) were partitioned in mixtures of PEG (3.6% w/vol; average molecular weight 20 kDa) and dextran (5.5% w/vol; average molecular weight 500 kDa). Partition coefficients were determined by measuring the absorbance of protein (280 nm) in each phase. **(B)** CZE traces analyzing mixtures of BCA and MYO prior to ATPP (bottom) and protein recovered from the dextran-rich phase after ATPP (top). After partitioning, the dextran-rich phase was dialyzed against Tris-Gly buffer to remove $NBu_4^+DS^-$. Protein was purified from dextran by precipitating the protein by adding aqueous ammonium sulfate, collecting the precipitate by centrifugation, and dissolving the pellet in Tris-Gly. The running buffer used in CZE consisted of Tris-Gly buffer containing 100 μM 4-carboxybenzenesulfonamide (**1**); association of **1** to BCA results in a shift in mobility by ~1 $cm^2\ kV^{-1}\ min^{-1}$ and improves the resolution of BCA from MYO.

**Fig. 8**


A
BCA
MYO
partition coefficient (dextran$_{aq}$ / PEG$_{aq}$)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
no surfactant
NBu$_4^+$Cl$^-$
Na$^+$DS$^-$
NBu$_4^+$DS$^-$
initially denatured by Na$^+$DS$^-$, followed by addition of NBu$_4^+$Cl$^-$
treatment prior to partitioning (at 22 °C)
B
DMF
MYO
BCA + 1
absorbance (214 nm)
dextran-rich fraction (after ATPP with NBu$_4^+$DS$^-$)
starting material (before ATPP)
-2
0
2
4
6
8
10
12
14
16
18
20
22
mobility (cm$^2$ kV$^{-1}$ min$^{-1}$)

While partition coefficients for BCA and MYO were similar in solutions without surfactant, or in solutions of $Na^+DS^-$, the partition coefficients were different by a factor > 3 when mixtures contained $NBu_4^+DS^-$. MYO was converted to MYO-$DS^-_n$ in solutions of $NBu_4^+DS^-$, and partitioned more favorably into the PEG-rich phase, while BCA remained and partitioned in its native form. These results suggested that these proteins can be separated by ATPP with the use of $NBu_4^+DS^-$.

We demonstrated the separation of BCA and MYO. We diluted a mixture of BCA and MYO (1 mg/mL in each protein) into a solution of 10.0 mM $NBu_4^+DS^-$. After partitioning the proteins in a mixture of 3.6% PEG and 5.5% dextran, we analyzed protein recovered from the dextran-rich phase. The CZE traces in Fig. 8B showed that the dextran-rich phase was enriched in BCA by a factor of ~5 compared to the initial mixture. This demonstration suggests that the selective formation of protein$^D$-$DS^-_{n,sat}$ in $NBu_4^+DS^-$ may, in some instances, provide a useful approach to the separation of proteins. This approach is most likely to be effective when the protein of interest is resistant to denaturation by $C^+DS^-$ (e.g., BCA in solutions of $NBu_4^+DS^-$), and impurities denature readily.

## CONCLUSIONS

**The cation influences the rate of denaturation of BCA by $DS^-$ through its influence on the cmc of $DS^-$.** This study provided a set of observations demonstrating the influence of $NR_4^+$ on the formation of BCA$^D$-$DS^-_{n,sat}$ from $DS^-$ and native BCA. SurfCE showed that variation of the cation, from $Na^+$ through increasingly hydrophobic $NR_4^+$, results in decreasing amounts of BCA$^D$-$DS^-_{n,sat}$ formed in reactions performed in

capillaries (windows of observation of 2 - 3 min., Figures 1 and 3). The underlying reason for the SurfCE results was the influence of $C^+$ on the cmc of $C^+DS^-$, and the dependence of the kinetics of denaturation of BCA on the concentration of monomeric $DS^-$. At concentrations below the cmc, rates of formation of $BCA^D$-$DS^-_{n,sat}$ increased sharply with the concentration of $DS^-$ (the rates of denaturation were proportional to $10^{[DS^-]}$) but were independent of $C^+$; these data indicate that the cation is a spectator to the interaction of $DS^-$ with BCA below the cmc. Rates of formation of $BCA^D$-$DS^-_{n,sat}$ did not increase with the concentration of $C^+DS^-$ above the cmc, but showed a wide range (a factor > $10^4$) in rates at 10.0 mM $C^+DS^-$, determined by the differences in values of the cmc of $C^+DS^-$. These data show that the cation can determine whether BCA is kinetically stable in solutions of $DS^-$ above the cmc (and therefore whether it remains in its native form is denatured by $DS^-$).

**The rate-limiting step in the denaturation of BCA by $DS^-$ involves interaction with monomeric $DS^-$.** Analysis of BCA in solutions of $Na^+DS^-$ and $NR_4^+DS^-$ allowed us to characterize the importance of the cmc of $C^+DS^-$ on the kinetics of denaturation. At concentrations of $C^+DS^-$ up to the cmc, BCA denatured with rates that were independent of $C^+$ and increased with the concentration of $DS^-$. Rates were, however, approximately constant at concentrations beyond the cmc. The kinetics showed that monomeric $DS^-$, and not micelles of $C^+DS^-$, determine the rates of denaturation *for BCA*.

The use of $C^+$ thus provided information relevant to the mechanism of denaturation of BCA. $NBu_4^+$ facilitates the assembly of $DS^-$ into micelles, but does not accelerate reactions of $DS^-$ with BCA. Although micelle formation is important to protein-$DS^-$ interactions – complexes of denatured protein and $DS^-$ consist of micellar

aggregates of $DS^-$ – the denaturation of BCA begins with interaction with monomeric $DS^-$.

**The cation is a potentially useful "knob-to-turn" in methods for the analysis and purification of proteins.** The effect of $NR_4^+$ on the formation of protein-$DS^-_n$ in SurfCE varied among the eight proteins tested. The results in solutions of $NBu_4^+DS^-$ distinguished proteins that were kinetically stable (BCA and CPK) from those that denatured rapidly (e.g., MYO). The results identified the cation as a component of protein-$DS^-$ interactions that can be exploited in methods for separating proteins, as illustrated in the use of $NBu_4^+DS^-$ in ATPP to separate MYO and BCA. Variation among proteins in the rates of denaturation by $NBu_4^+DS^-$ enables the selective denaturation of proteins in mixtures, and can be used to improve the results of existing methods for purifying proteins (e.g., ATPP).

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**Supporting Information Available:** Chemicals used; experimental protocols; additional figures (Figures S1 - S20).

**REFERENCES.**

(1) The electrophoretic migration of BCA in SDS-PAGE is consistent with its molecular weight (29.1-kDa). Based on stoichiometries of binding that are typical for proteins in SDS-PAGE, we assume that the formation of $BCA^D$-$DS^-_{n,sat}$ involves the association of ~150 equivalents of $DS^-$ (i.e., $n$ ~ 150, estimated from the binding of ~1.4 g of $Na^+DS^-$ for every 1 g of protein).

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(92) Details of the experimental procedures are available in the Supporting Information.

(93) One reason for why values of cmc determined with pyrene are lower than those determined with 2-naphthalenecarbinol is that pyrene is more hydrophobic than 2-naphthalenecarbinol. Pyrene may associate with $DS^-$ or induce micelle formation (resulting in changes in its fluorescence) at concentrations of $DS^-$ slightly lower than the cmc determined by 2-naphthalenecarbinol.

(94) Although this contribution leads to a decrease of ~15% in the concentration of $NBu_4^+DS^-$ by the end of the reaction, there was good agreement between the data and the line obtained by fitting to a first-order exponential decay ($R^2 = 0.99$).

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(98) Peaks for CPK shifted by less than 5 $cm^2\ kV^{-1}\ min^{-1}$ when analyzed in $Na^+DS^-$ and $NR_4^+DS^-$, and suggested the association of a small number of equivalents of $DS^-$. The mobilities of derivatives of CPK acetylated at Lys ε-$NH_3^+$ residues

suggested that the formation of CPK-$DS^-_n$ in SurfCE takes place with an increase of less than two units of negative charge.

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